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TRANSLATION OF AVIAN SARCOMA VIRUS RNA IN XENOPUS LAEVIS OOCYTES

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Summary: Evidence for the synthesis and processing of Pr76 (precursor to group-specific antigens p27, p19, and p12/15, upon injection of avian sarcoma virus 70S or 35S RNA into Xenopus oocytes has been presented. Further, we show that $tRNA^{trp}$ primer, bound to 35S RNA, does not block translation of virion RNA under these conditions.

The 35S virion and 35S messenger RNA's of avian sarcoma virus contain four genes, which have been ordered as 5' gag-pol-env-src-3' (1). The gag gene codes for a protein of 76,000 daltons (Pr76), which is subsequently processed to give rise to the major internal virion proteins (p27, p19, p15, and p12), the so-called gs antigens (2). The characteristics of these and other virus proteins have been described (2). Several groups have independently translated 35S RNA in in vitro systems such as Krebs ascites and reticulocyte lysates (3-6). The limitation of most cell-free translation systems, however, appears to be the absence of post-translational cleavage of precursor molecules to give rise to appropriate final products.

Several species of tRNAs are associated with the 60-70S complex of virion RNA. One of these which has been shown to be specific for trytophan ($tRNA^{trp}$), is located near the 5' end of 35S RNA subunit, and functions as a primer for DNA synthesis (7). In avian sarcoma virus-infected cells the 35S RNA serves as a messenger and also is encapsidated in the virion. In an effort to investigate the regulation of 35S by bound $tRNA^{trp}$ primer of avian sarcoma virus, we have decided to use the Xenopus laevis oocyte translation system. The major advantages of this system, as opposed to the cell

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free systems are: 1) the requirement for relatively small quantities of exogenous mRNA and 2) the injected mRNAs are translated for longer periods of time (8).

In this brief communication we will describe our results which show 1) the synthesis and post-translational processing of Pr76 after microinjection of avian sarcoma virus 35S subunits or aggregated 60-70S virion RNA, confirming earlier work on avian myeloblastosis virus RNA (9) and 2) that bound $tRNA^{trp}$ primer does not appear to affect the capacity of 35S virion RNA to direct protein synthesis.

MATERIALS AND METHODS

Cells and Viruses: Avian sarcoma virus, (ASV) Prague C, was propagated in chick embryo fibroblasts (CEF) (10). The cells prepared from 11 to 12 day-old embryos (Spafas, Connecticut), were grown in medium 199 as previously described (11).

Immunoprecipitation and Gel Electrophoresis of Viral Proteins: Anti-viral anti-serum was raised in New Zealand white rabbits essentially as described previously (12). Virion 70S RNA was prepared from ASV-PrC or PrB virus and it was resuspended at a concentration of 250 µg in oocyte injection buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris-HCl pH 7.6). X. laevis frogs were obtained from Charles W. Fletcher, Hampstead, Md. For each RNA sample 10-12 oocytes at stage V-VI (8) were injected. Oocytes were incubated at 22° for the periods indicated below in 100 µl modified Barth's solution (13) containing 100-150 µCi ^{35}S-methionine (300 Ci/mmole). ^{35}S-methionine labeled PrB virus was prepated as previously described (2). For analysis of intracellular viral proteins, PrB virus-infected chick embryo fibroblasts were labeled with 100 µCi/ml ^{35}S-methionine in methionine-free Eagle's medium containing 10% dialyzed calf serum. Immunoprecipitation was carried out according to Kessler using Cowan I strain Staphylococcus aureus as an immune absorbant (14). Ooocytes and cells were washed in Barth's medium and PBS respectively and lysed in 0.5-1 ml NET (0.1 M NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.4) containing 0.5% NP-40. Cellular debris was removed by centrifugation. All samples were first treated with 5-15 µl of preimmune serum, followed by precipitation with 2-5 µl of immune serum for 30 minutes at 4°C, using the bacterial absorbant for both steps. After absorption, bacteria were washed 3 times in 0.05% NP-40 NET buffer, resuspended in 10 µl of SDS-gel sample buffer (3% SDS, 0.01% phenol red, 1% β mercaptoethanol, 50 mM Tris-HCl pH 6.8, 10% glycerol) and boiled for 3 minutes. Bacteria were removed by centrifugation and the resulting supernatants were analyzed on 17.5% SDS-acrylamide gels (15). The ^{35}S-labeled protein bands were located by fluorography (16).

Assay for the Release of tRNA Primer: $tRNA^{trp}$ primer which is bound to the virion RNA, was specifically labeled with ^{32}P by initiating complementary DNA synthesis in the presence of dCTP analogue, Ara-CTP, essentially as described by Taylor et al. (17). The reaction mixture containing 50 mM Tris-HCl pH 7.4, 50 mM KCl, 10 mM dithiothretol, 10 mM $MgCl_2$, 0.05% NP-40, 10 µM dGTP, dTTP

(P-L Biochemicals), β-D-furanosyl arabinoside triphosphate (Ara CTP), 600 μCi/ml {α-^{32}P} dATP (NEN) and purified avian sarcoma virus, was incubated at 37°C for 1 hour. The 70S RNA-^{32}P-cDNA product was separated from the free isotope on sucrose gradient, extracted with phenol, and concentrated by ethanol precipitation. The complexes were resuspended in oocyte injection buffer and heated to the temperatures as indicated in Figure 2. Release of the {^{32}P} cDNA-tRNA covalent complex from the 70S RNA was monitored by electrophoresis in 10% acrylamide gels using a tris-borate buffer system. {^{3}H} E. coli tRNA was included in each sample as an internal marker.

RESULTS AND DISCUSSION

Xenopus laevis oocytes were injected with avian sarcoma virus RNA (250 μg/ml, 50 ml/oocyte) from Prague C (PrC) as previously described (18). The oocytes were incubated with ^{35}S-L-methionine-containing medium for 5, 24, or 48 hours. The proteins were isolated from oocytes and radio-labeled proteins were immunoprecipitated with rabbit antiserum directed against Triton X-100 disrupted virions. The immune complexes were separated by addorption to formalin-fixed S. aureus cells (14) and then analyzed by polyacrylamide gel electrophoresis.

The results presented in Figure 1 show that virus specific proteins can be translated in oocytes in response to injected viral RNA, as evidenced by the size of the proteins which are precipitated by immune serum. No bands corresponding to viral proteins are visible in samples treated with preimmune serum (data not shown).There are no immunporecipitable proteins from uninjected controls (Fig. 1c and d). Five hours after injection the major band correspond to Pr76 (data not shown). In addition to pr 76, p27, two other high molecular weight viral proteins with molecular weights of approximately 64,000 and 58,000 daltons have appeared after 24 hours (Fig. 1a-c). This cleavage pattern is similar to that seen in infected cells (2). Analysis of 48 hour-labeled oocyte lysates revealed additional bands corresponding to viral proteins p19 and p15/12 (Figure 1, e and f). (Protein p15/12 appears faintly in the photograph, but is clearly visible on the autoradiogram). The band labeled H results from non-specific trapping by immunoglobulin heavy chains.

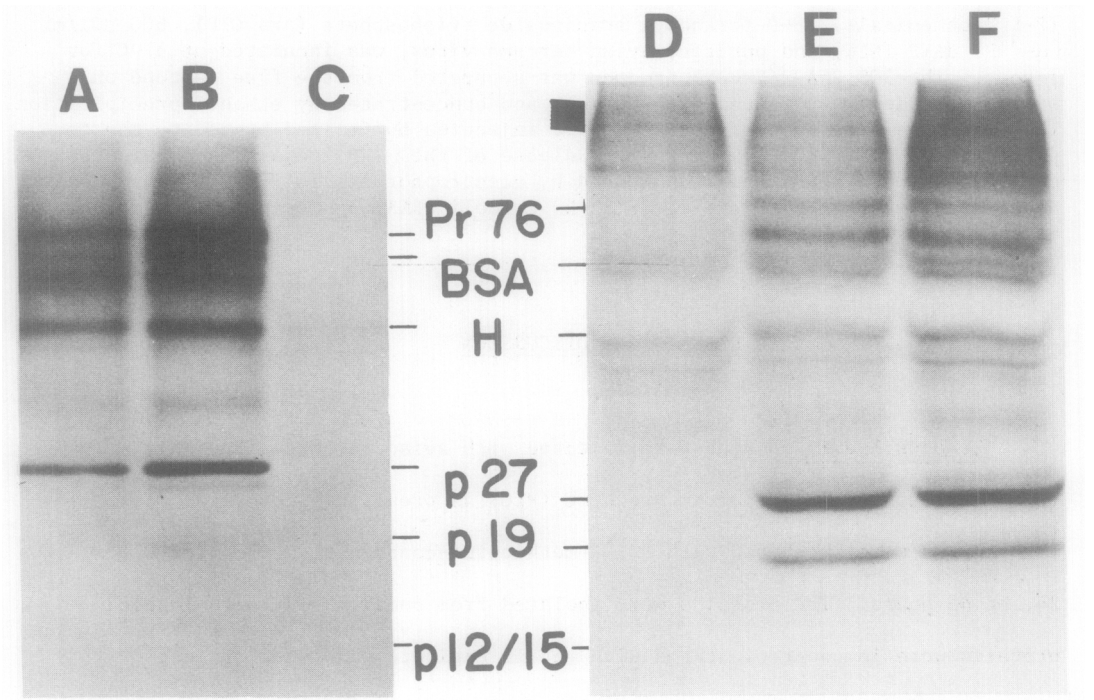

Figure 1. Autoradiogram of SDS-polyacrylamide gel electrophoresis of ^{35}S-methionine labeled proteins precipitated from X. laevis oocyte lysates. a-c, oocytes incubated for 24 hours. Viral RNA heated for 3 minutes at (a) 100°C, (b) 50°C prior to injection; (c) uninjected control; d-f, oocytes incubated for 48 hours; (d) uninjected or viral RNA heated (e) 50°C or 80°C (f) prior to injection.

These results support the notion that the group-specific proteins (gag gene products) are coded by 35S RNA (3,4,9). That the oocyte system, in contrast to the cell-free systems, has the ability to process precursor proteins into the expected final products is also evident from these data.

Previous studies from other laboratories indicated that the Tm for dissociating bound $tRNA^{trp}$ from 35S RNA is 63°C in 0.01 M Tris-HCl-0.01 M EDTA (18). ASV RNA heated at 50°C, 80°C or 100°C in 88 mM NaCl buffer prior to injection did not reveal any major differences in its ability to direct protein synthesis although structural changes in the RNA molecule were evident. We found, in agreement with published results (19), that approximately 30% of the 60-70S RNA dissociated into 35S RNA subunits at 50°C and 80°C all the RNA sedimented at 35S. Since the injection buffer contains 88 mM NaCl the following

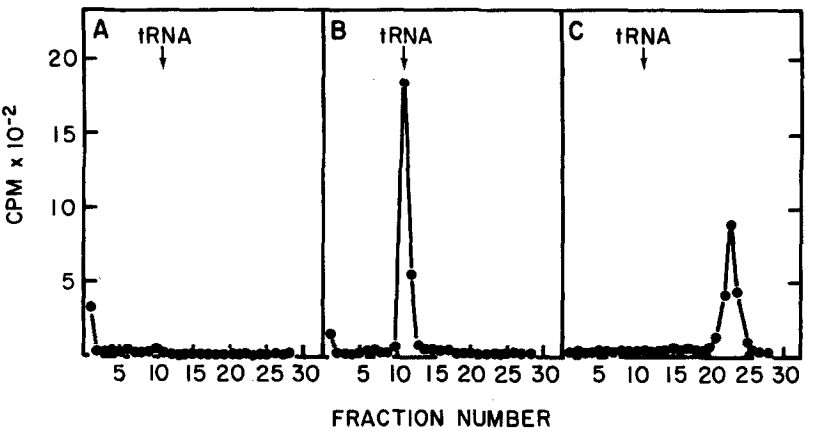

Figure 2. Polyacrylamide gel assay for the release of primer. (a) 60-70S RNA containing ^{32}P-labeled octamer-tagged primer run as a control; (b) complex heated at 80°C in 88 mM NaCl and (c) complex digested with 0.2 M NaOH at 100°C for 3 minutes prior to analyzing on the gel.

experiment was performed to prove that under the conditions of melting all the tRNA primer was removed from 60-70S RNA. Purified ASV was incubated in an endo genous reaction containing ^{32}P labeled dATP as described in the Methods section and ara-CTP replaced dCTP. In the presence of this analogue the major product is an oligo-nucleotide of 8 residues (octamer) covalently linked to the primer (16). After the reaction, viral RNA containing the radio-labeled primer was isolated, heated at 80°C in 88 mM NaCl, and analyzed in polyacrylamide gels. Under these conditions the tRNA primer which is tagged with ^{32}P-labeled octamer was completely dissociated from 60-70S RNA as evidenced by a discrete peak of radioactivity comigrating with E. coli tRNA marker (Fig. 2b). In the unheated control the radio-activity remained at the origin indicating that the primer is associated with 60-70S RNA (Fig. 2a). Digestion of the product with alkali, which removes the RNA molecule of the hybrid, caused the DNA to migrate at the expected position for an octamer (Fig. 2c). From these results we conclude that the conditions used to release the primer are satisfactory. Injection of the RNA samples heated at these temperatures did not reveal any major variations in the levels of translation (Fig. 1). However we

observed that RNA heated at 50°C was sometimes better in its capacity to translate than that heated at more than 80°C. This is probably due to random breaks introduced in the RNA molecule at higher temperatures. In addition, we consistently found that RNA which was not heated had translation ability similar to heated samples (data not shown).

These results indicate that under the conditions used here for translation, virion 35S RNA as well as the 60-70S complex can be used as a template for the synthesis of viral proteins. In addition, the data presented here suggest, but do not prove, that initiation of protein synthesis is internal to the primer binding sequence, since the presence of primer has no apparent effect on translation*. If this is true, then the results may mean that the primer has no role in distinguishing 35S mRNA from 35S virion RNA. In support of this argument data presented by Canaani and Duesberg (20), indicated that the RNA from immature virions does not contain bound primer, but, after incubation at 37°C, complete 60S-70S-primer complex can be generated.

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* In agreement with these data, amino acid sequence data indicated that the possible initiation AUG codon beginning at nucleotide residue 83 (which is on the 5' end of $tRNA^{trp}$ binding site) does not serve as an initiation codon for Pr76 (R. Eisenman, personal communication).

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