THE TRANSLATION OF VACCINIA VIRUS MESSENGER RNA IN ANIMAL CELL-FREE SYSTEMS

F. FOURNIER*, D.R. TOVELL*, M. ESTEBAN, D.H. METZ*, L.A. BALL and Ian M. KERR
National Institute for Medical Research, Mill Hill, London NW7 1AA, England

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1. Introduction

Cell-free systems from animal cells in which added viral messenger RNA's (mRNA's) are translated have proved useful in studies on eukaryotic protein synthesis and its control. Of the viruses which contain their own transcriptases for the synthesis of viral mRNA, however, only with reovirus has it been possible to synthesise *in vitro* sufficient intact mRNA for its translation to be studied in this way [1–3]. Vaccinia is a large DNA virus which contains an RNA polymerase activity associated with the virus cores [4]. The core polymerase, prepared *in vitro* from purified virus, will effect the synthesis of RNA having properties similar to the viral mRNA present at early times in vaccinia-infected cells [4]. This RNA is a mixture of species, the sizes of which are consistent with their coding predominantly for individual viral proteins rather than for very large precursor molecules as is the case with the picornavirus RNA's [5, 6]. Furthermore these RNA species unlike the reovirus mRNA's have poly A at their 3'-ends [7]. Thus these presumptive vaccinia mRNA's appear to resemble animal cell messengers more than do either of the other viral mRNA species most readily available for study – those of reo- and picornaviruses. As yet, however, no direct demonstration of messenger function has been reported for this vaccinia RNA. Here we describe its translation in two mouse cell-free systems.

2. Materials and methods

Vaccinia virus (strain WR) was grown and purified essentially according to Joklik [8] as previously described [9]. The method of Kates and Beeson was used in the preparation of the virus cores [4]. The reaction mixture for RNA synthesis contained: 2×10^{11} cores; 10 μmoles ATP; 5 μmoles each of CTP, GTP and UTP; 10 μCi [^{3}H]UTP (40 Ci/mmole); 20 μmoles $MgCl_2$; 40 μmoles 2-mercaptoethanol; 100 μmoles phosphoenolpyruvate; 100 μg pyruvate kinase; 250 μg Macaloid [10]; 200 μmoles Tris-HCl, pH 8.5; in a total volume of 4.0 ml. Incorporation of radioactivity from [^{3}H]UTP into an acid-insoluble, alkali and RNAase-labile product was completely dependent upon the presence of ATP, GTP and CTP. The mixture was incubated at 33° for 3 hr during which incorporation was almost linear, then centrifuged at 30,000 *g* for 30 min to remove the cores and the Macaloid. The supernatant was treated with 10 mM EDTA and 1% (w/v) sodium dodecyl sulphate (SDS) at 37° for 5 min and the mixture was layered onto a 15–30% sucrose (w/v) gradient in 10 mM Tris-HCl pH 7.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS and centrifuged in the SW 27 rotor at 70,000 *g* for

* Present addresses: F.F., Institute National de la Santé et de la Recherche Médicale, Unité de Recherches sur les Virus U. 43, Hopital Saint-Vincent-de-Paul, Paris XIV. D.R.T., Dept. of Microbiology and Immunology, Queens University, Kingston, Ontario, Canada. D.H.M., Research Division of Infectious Diseases, Childrens Hospital Medical Center, 300 Longwood Avenue, Boston, Mass. 62115, USA.

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20 hr at 20°. Fractions were collected and aliquots were taken for measurement of acid-insoluble radioactivity. The fractions containing the greater part of the RNA were pooled and precipitated by the addition of 2.5 vol of ethanol. After storage overnight at −20°, the RNA was sedimented, washed once with ethanol, dried in vacuo and dissolved in sterile water prior to storage in small aliquots at −20°. The concentration was determined from the absorbance at 260 nm assuming that an absorbance of 1.0 is given by a solution of 40 μg/ml. The final yield from such a preparation was of the order of 500 μg.

Ribosome and cell sap components for the Krebs cell-free system and the L-cell extracts were prepared as previously described [11, 12]. In each case the endogenous protein synthetic activity was reduced by preincubation of the post-mitochondrial supernatant fraction. The response of the Krebs cell-free system to added vaccinia RNA was assayed in 30 μl mixtures as for encephalomyocarditis virus (EMC) RNA [13] except that incubation was for 60 min at 30° rather than 37°. For the L-cell-free system each 50 μl reaction mixture contained the following (final conc.): 30 mM Tris-HCl, pH 7.6, 7 mM 2-mercaptoethanol, 3.5 mM Mg acetate, 40 mM KCl, 1 mM ATP, 0.1 mM GTP, 10 mM creatine phosphate, 0.16 mg/ml creatine kinase, 5 μCi/ml ^{14}C-amino acid mixture (> 45 mCi per milliatom carbon), 40 mM each of the amino acids not present in the ^{14}C-amino acid mix and 10 to 15 μl of L-cell extract [11, 12]. For the preparation of material for gel electrophoresis, the assays were scaled up twenty-fold and the ^{14}C-amino acid mixture was replaced by [^{35}S]methionine (20 to 30 Ci/mmole) plus the "other nineteen" non-radioactive amino acids. 50–100 μl aliquots of the cell-free system product were treated with 2% SDS, 8 M urea and 0.5 M 2-mercaptoethanol at 100° for 2 min prior to electrophoresis on 10% polyacrylamide gels in the presence of SDS [14]. The preparation and electrophoresis of [^{35}S]methionine-labelled polypeptides from vaccinia-infected cells has already been described [9, 15]. Gels were sliced longitudinally and subjected to autoradiography.

All radiochemicals were purchased from the Radiochemical Centre, Amersham, Bucks, England.

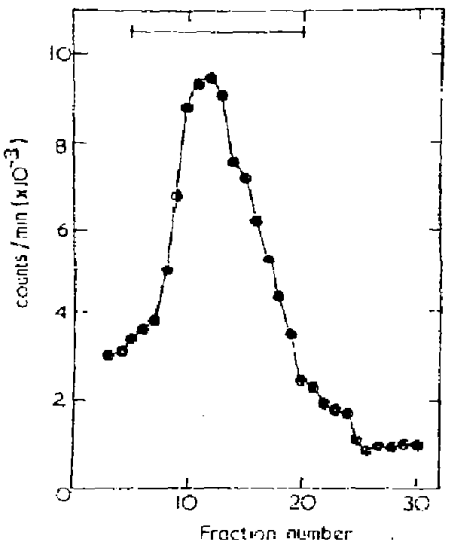

Fig. 1. Sucrose gradient sedimentation of ^{3}H-labelled vaccinia RNA synthesised *in vitro* with the core polymerase from purified virus. Sedimentation was from left to right. The bar indicates the fractions that were pooled and precipitated to yield the RNA used in the cell-free systems.

3. Results and discussion

3.1. *Characterisation of the vaccinia RNA and conditions for its translation*

The viral RNA synthesised and released by the isolated cores sediments in a sucrose gradient as a broad peak at about 12 S (fig. 1) [4]. The sedimentation behaviour of this RNA is very similar to that of early vaccinia mRNA seen in infected cells shortly after infection [4, 9]. Electrophoresis of the RNA in polyacrylamide gels yielded at least 10 discrete species of 3×10^4 to 10^5 in apparent molecular weight on the basis of comparison with marker RNA's (fig. 2).

The conditions for optimal stimulation of amino acid incorporation by this RNA in the Krebs cell-free system were very similar to those for EMC RNA [11, 13], maximum incorporation being obtained with 4 to 6 mM Mg^{2+} in the presence of 50 mM K^+. Incubation at 30° was as good or better than at 37°. Most of the incorporation occurred during the initial 20 to 30 min at either temperature. Typically a 2 to 5-fold stimulation was observed at a saturating RNA concentration of about 60 μg/ml. With the L-cell extracts, maximum incorporation of amino acids in

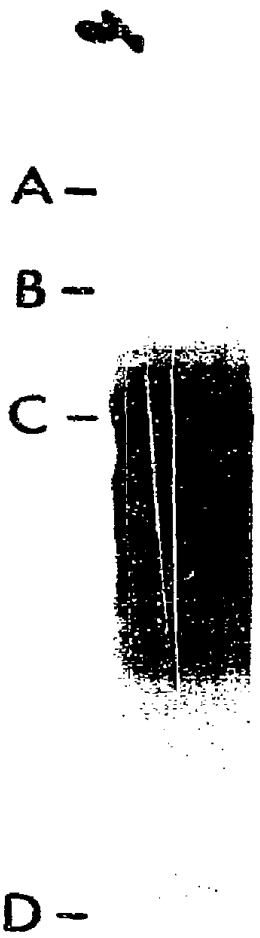

Fig. 2. Vaccinia RNA was labelled with [^{14}C]UTP, isolated as described in the text and an amount corresponding to 7,000 cpm was applied to a 2% polyacrylamide gel containing 0.5% agarose and subjected to electrophoresis at 100 V (4.5 mA per gel) for 1.5 hr [23]. A, B, C, and D indicate, respectively, the positions to which Encephalomyocarditis virus RNA (37 S) and 28 S and 18 S ribosomal RNA and 4 S tRNA from mouse Krebs ascites tumour cells included as markers migrated. These markers did not affect the distribution of the radioactive species of vaccinia RNA. After staining with methylene blue to show the unlabelled markers, the gels were dried and subjected to autoradiography for 5 weeks.

response to the added vaccinia RNA occurred at 3.5 mM Mg^{2+}, 40 mM K^+ and 40 μg/ml of RNA and was essentially complete after 60 min at 30°.

3.2. *Analysis of the polypeptide products synthesised in response to the vaccinia RNA*

In the intact vaccinia virus-infected L-cell a very rapid inhibition of host protein synthesis occurs immediately after infection. Thus by 20 to 60 min post infection the vast majority of protein synthesis is virus mediated [9, 15–17]. This synthesis is thought to occur on mRNA's produced by the transcriptase of the infecting parental virions. It should be emphasized, however, that the synthesis of the different viral polypeptides is under very fine control, their relative amounts varying with time post infection [9, 15, 16 and fig. 3, gels 4 and 5]. It is not yet known for how many of these polypeptides the isolated core polymerase from purified virus synthesises mRNA *in vitro*. An estimate for this can be made, however, from the fact that about 7% of the DNA is transcribed [4] to yield messages theoretically equivalent to approx. 500,000 molecular weight units of protein or 10 to 25 polypeptides of 20,000 to 50,000 in molecular weight. The products synthesised in the cell-free system and the intact cell were compared by electrophoresis on SDS polyacrylamide gels (fig. 3, gels 1 to 6). It is clear that while at least 14 discrete polypeptides are formed in the cell-free system in response to the vaccinia mRNA not all of these correspond to polypeptides present in the intact cell. There is some coincidence, for example, the pair of polypeptides at approx. 30,000 molecular weight are particularly characteristic, but also significant differences (figs. 3 and 4). Immunological identification of the products has not yet been possible. This may reflect a failure in the phosphorylation [18] or glycosylation of proteins in the cell-free systems, or the fact that the sera used contained antibody to "late" rather than "early" viral proteins. A failure in glycosylation or in post-translational cleavage [19, 20] in the maturation of some of the polypeptide products in the cell-free system could also explain the fact that not all of these products correspond exactly in electrophoretic mobility to those present in the intact infected cell (fig. 3, gels 1 to 6). On the other hand the two mouse cell-free systems used here are known to be capable of

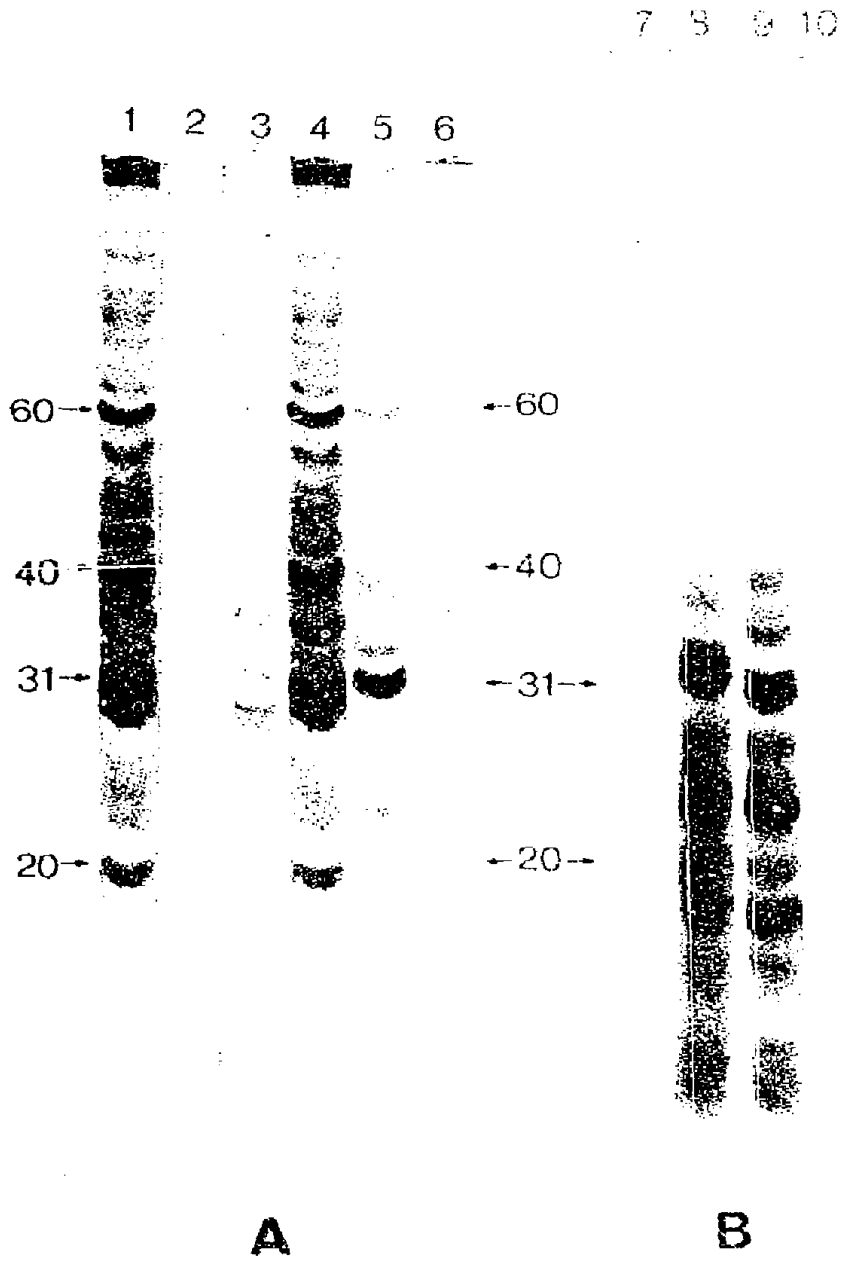

Fig. 3. Polyacrylamide gel electrophoresis of [^{35}S]methionine labelled polypeptides synthesised in vaccinia-infected cells and in cell-free systems in response to vaccinia RNA. Infected L-cells pulse labelled from 20–40 min (gels 1 and 4) and 60 to 80 min (gel 5) post infection. Uninfected cells pulse labelled for 20 min (gel 6). Krebs cell-free system with (gels 3 and 9) and without (gels 2 and 10) vaccinia RNA. L-cell extracts with (gel 8) and without (gel 7) vaccinia RNA. Gels 1 to 6 and 7 to 10 were run on separate occasions. The approx. molecular weights ($\times 10^{-3}$) of some of the infected-cell polypeptides are shown.

the translation of added mRNA's in the form of mouse globin and EMC RNA's [12, 20–22]. Moreover, in preliminary experiments, fingerprints of tryptic peptides of the vaccinia RNA-stimulated products formed in both the Krebs and L cell-free systems showed definite similarities with those from vaccinia polypeptides formed at 20 to 60 min but not 180 min post infection in the intact cell. They were, of course

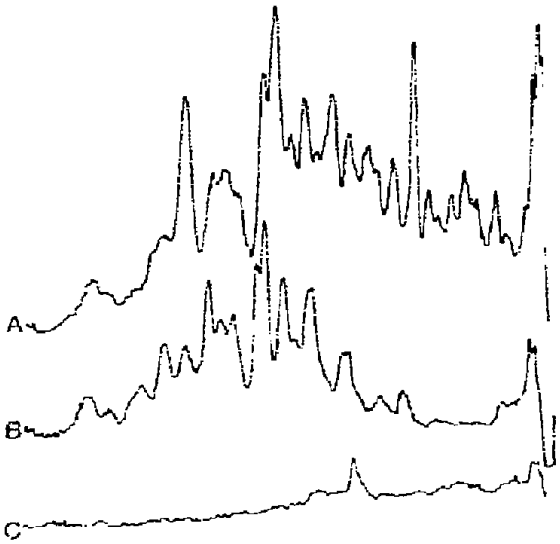

Fig. 4. Comparison of the polypeptides formed in response to vaccinia RNA in the Krebs cell-free system with early vaccinia polypeptides from infected L-cells. Analysis of the [^{35}S] methionine-labelled polypeptides was by electrophoresis on SDS-polyacrylamide gels. The figure presents densitometer tracings of autoradiograms of the dried gels with the origins to the right. A) Vaccinia-infected cell polypeptides pulse labelled 20 to 40 min post infection. B and C) Polypeptides from cell-free systems incubated with (B) and without (C) vaccinia RNA.

clearly different from those of the products formed in response to EMC RNA in these systems. Finally, the same polypeptide products appear to be synthesised in response to the vaccinia RNA in the two completely distinct cell-free systems from L and Krebs cells (fig. 3 gels 7 to 10). It is reasonable to conclude, therefore, that it is the added vaccinia mRNA that is being translated in the two systems, for it seems highly unlikely that identical polypeptide products would be produced by an artefactual vaccinia mRNA-mediated stimulation of the translation of endogenous mRNA of L cells in the one case and Krebs cells in the other. Accordingly, while absolute proof awaits the results of a more detailed study, the data presented here clearly indicate that the vaccinia core polymerase is making RNA which is not only complementary to vaccinia DNA [4] but is also active messenger capable of being translated in appropriate cell-free systems.

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Addendum: Similar results have recently been reported in [24].