TRANSLATION OF FRAGMENTED VIRAL RNA IN VITRO

Initiation at multiple sites

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Received 18 January 1979

1. Introduction

Translation in eucaryotic cell-free systems has recently been used to identify hitherto unknown genetic products, mainly of RNA viruses (e.g. [1–6]). Some of these studies involve translation of sub-genomic mRNAs [2–6], which may be contaminated with the random fragments of genomic RNA that are present in most viral preparations. In other cases, genomic RNA is intentionally fragmented in an attempt to activate internal genes which are not translated from intact RNA. Most of the products from such degraded RNA are 'early quitters' which are initiated at the same point as with intact RNA, but terminate prematurely at a fragmentation site [7,8]. Products coming from other initiation sites are assumed to be read from contaminating mRNAs or from activated internal genes; however, the physiological relevance of such products depends on the ability of the cell-free system to select only 'correct' ribosome binding sites, regardless of the state of the added RNA. I show here that under normal conditions reticulocyte ribosomes can initiate protein synthesis at many apparently spurious sites on partially degraded viral RNA.

The RNAs used in this work were from cowpea mosaic virus (CPMV) and tobacco mosaic virus (TMV). CPMV is a multicomponent virus with two RNAs of M_r 1.37×10^6 and 2.02×10^6 [9]. Both of these RNAs are polyadenylated but not capped [10]; they each code in vitro for large proteins which account for all (larger RNA) or nearly all (smaller RNA) of their coding capacities [1,11]. TMV RNA is capped [12] but not polyadenylated and codes in vitro for a major product of M_r 110 000 (previously estimated as 130 000–140 000) and a readthrough protein of M_r 160 000 [1,13,14]. The latter accounts for ~75% of the coding capacity.

2. Materials and methods

Translation in the mRNA-dependent reticulocyte lysate, labelling with f[^{35}S]Met-tRNA$_f$ and SDS gel electrophoresis were as in [1,15]. Peptide mapping by partial proteolysis in SDS was by an adaptation of the method in [16]. Gels were dried onto dialysis membrane without fixing or staining, bands were located by autoradiography, and individual bands or tracks cut out after softening the gel with steam. Gel fragments were swollen in 10% glycerol, 0.125 M Tris (pH 6.8), 1 mM EDTA, 0.1% SDS containing 0.1 mg/ml chymotrypsin (or pronase) for 1 h at 37°C, using just enough buffer to allow complete swelling. They were then placed on top of a 20% gel with 5% stacking gel, and electrophoresed overnight. The 20% gel was fluorographed [17].

TMV *vulgare* was a gift from Dr J. Butler, and CPMV from Dr A. Van Kammen. RNA was isolated by heating the virus to 65°C in 1% SDS, 0.1 M NaCl, 1 mM EDTA, 10 mM Tris (pH 7.5), followed by either phenol extraction or sucrose gradient centrifugation of the RNA. m^{7}GTP was obtained from PL Biochemicals, and added together with an equimolar amount of $MgCl_2$.

3. Results

3.1. *N-terminal analysis of products made from fragmented CPMV RNA*

Preparations of the larger CPMV RNA are often partially degraded, as judged by sucrose gradient analysis. Translation of such fragmented RNA in the reticulocyte system reproducibly yields a number of discrete products of M_r up to ~60 000 (fig.1a) which are not obtained with intact RNA. As part of a search for possible subgenomic mRNAs, the N-termini of these products were analysed. Translation was performed in the presence of f[^{35}S]Met-tRNA$_f$, which donates label only into the N-terminal position, the fMet residues being resistant to proteolytic removal [18]. The products were separated on an SDS–gel and then analysed by limited proteolysis in SDS [16]. In this procedure a strip of the first gel is soaked in protease-containing buffer and laid across the top of a second slab gel. The cleaved fragments are electrophoresed into the second gel and detected by fluorography. Translation products of different sizes which share a common N-terminus yield identical labelled fragments which comigrate on the second gel.

Figure 1b shows such an analysis of some of the major products. Unexpectedly, they all gave different N-terminal fragments, implying that they are read from different initiation sites. This result was not due to contamination with cellular mRNAs, or to varying accessibility of the products to protease, since when the experiment was repeated with [^{35}S]methionine as label all the products gave similar digestion patterns (data not shown), indicating that they share common sequences (and perhaps a common C-terminus). These characteristics of shared internal sequences but different N-termini were confirmed for several individual bands using digestion with pronase, papain, trypsin and chymotrypsin. Some examples are shown in fig.1c,d.

3.2. *N-terminal analysis of products made from fragmented TMV RNA*

Since the above results were unexpected, a similar analysis was made of the products obtained with fragmented TMV RNA. Figure 2a shows a typical result, which is strikingly different from that shown in fig.1b. Most of the small TMV products clearly share a common initiation site, which corresponds to

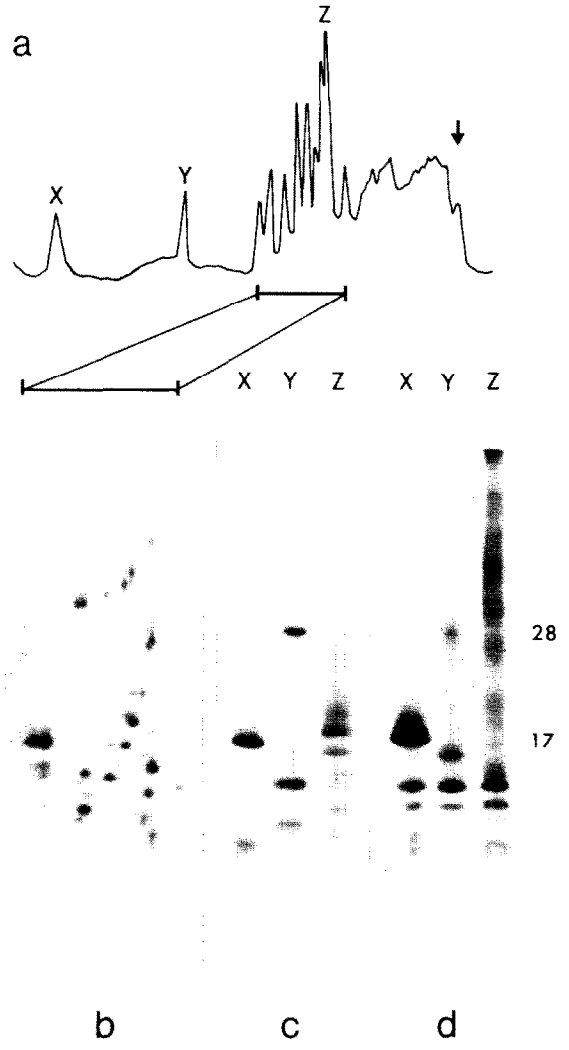

Fig.1. Limited proteolysis of CPMV products. (a) Densitometer tracing of an autoradiogram of an SDS–gel, showing [^{35}S]Met-labelled translation products from a partially degraded preparation of the larger CPMV RNA. Migration was from right to left, and the arrow indicates the translation product of intact RNA. (b) The same RNA preparation was translated in the presence of f[^{35}S]Met-tRNA$_f$ and the products separated as in (a). The indicated region of the gel was then treated with chymotrypsin and the digestion products analysed on a second-dimension gel as in section 2. Migration was from top to bottom; a fluorogram is shown. (c) Three individual products, labelled with f[^{35}S]Met-tRNA$_f$ and digested with pronase. (d) The same products as in (c), labelled with [^{35}S]Met and digested with chymotrypsin. The molecular weights of X, Y and Z were 17 000, 28 000 and 56 000, respectively.

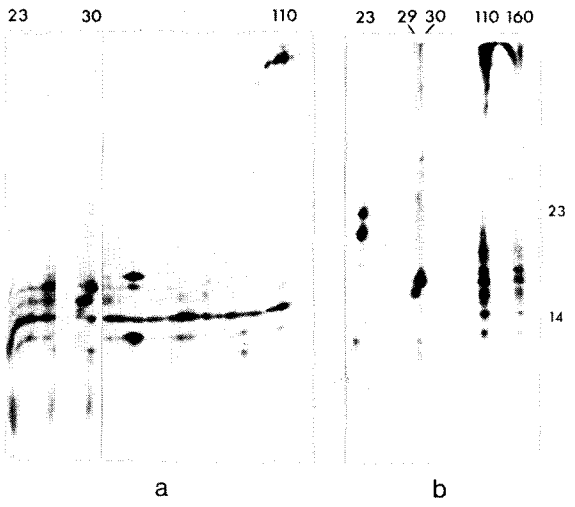

Fig.2. Limited proteolysis of TMV products labelled with f[^{35}S]Met-tRNA$_f$. Products from a partially degraded preparation of RNA were separated on an SDS–gel, digested with chymotrypsin and the peptides separated on a second-dimension gel as in fig.1. Numbers at the top refer to the $M_r \times 10^{-3}$ of the translation products; those at the side to the $M_r \times 10^{-3}$ of the digested peptides. (a) Total products. The pattern obtained after the first dimension was similar to those shown in fig.3. The region corresponding to the 30 000 mol. wt protein has been underexposed for clarity. (b) Individual bands (from different batches of RNA) analysed separately.

that used in intact RNA. Nevertheless, some products are derived from other sites. Figure 2b shows an analysis of three of the more prominent ones, of M_r 30 000, 29 000 and 23 000. These all appear to be read from different sites, though they share common tryptic peptides (A. R. Hunter, personal communication). The largest one has been observed in the wheat germ system [3,4]. It appears to be the product of a specific TMV gene, and is thought to be made from a capped subgenomic mRNA formed during infection, although it is also made in vitro from apparently random fragments of virion RNA [4]. Thus the initiation site for this protein may be a physiological one activated by fragmentation, though synthesis of the others may well be artefactual. In contrast, the genuine initiation site for TMV coat protein is not activated by random fragmentation of virion RNA [5].

3.3. *Effects of m^{7}GTP on synthesis of small TMV proteins*

It seemed possible that other artefactual initiation sites might be activatable in TMV RNA, but it was hard to pick out minor products coming from such sites in experiments like the one shown in fig.2a. However, TMV RNA is capped, and therefore synthesis from the normal site can be inhibited specifically by the cap analog m^{7}GTP [19]; products

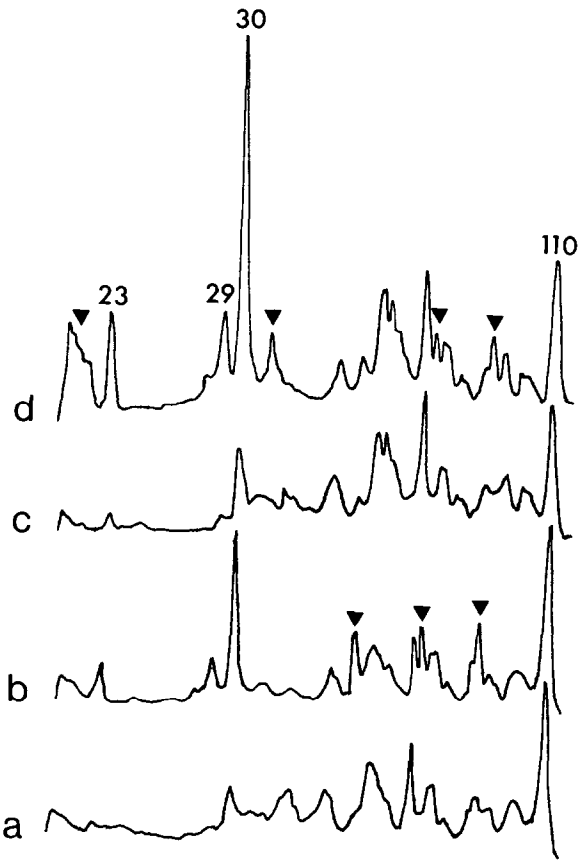

Fig.3. Effect of m^{7}GTP on translation of fragmented TMV RNA. Densitometer traces of a fluorographed SDS–gel are shown. Incubations contained: (a) Virion RNA fragmented with micrococcal nuclease (EC 3.1.4.7) (10 μg/ml, 1 mM $CaCl_2$, 1 min at 30°C); (b) as (a) with 0.25 mM m^{7}GTP; (c) fragments (~18 S) from another preparation of virion RNA; (d) as (c) with 0.5 mM m^{7}GTP. Protein synthesis was 80% inhibited in (b) and (d); approximately equal amounts of [^{35}S]Met-labelled protein were analysed in each case. Numbers indicate $M_r \times 10^{-3}$ of prominent bands. Arrows mark other proteins whose synthesis is resistant to m^{7}GTP.

coming from other (uncapped) sites should thus be detectable as gel bands whose intensity in unaffected by this inhibitor. Figure 3 shows the effects of cap analog on the product patterns obtained from TMV RNA fragmented either accidentally during purification or intentionally with micrococcal nuclease. The gel samples were adjusted so that they contained equal acid-precipitable radioactivity; thus m^7GTP-resistant bands appear with increased intensity in the samples labelled in the presence of the inhibitor.

The 30 000, 29 000 and 23 000 M_r products all appear to be made from uncapped RNAs, suggesting that they do not originate from contaminating capped subgenomic mRNAs. Besides these, a number of other products were reproducibly resistant to the effect of m^7GTP, though they were made in rather small amounts (fig.3). Thus several different initiation sites are recognized (albeit inefficiently) in degraded TMV RNA.

4. Discussion

The results presented here indicate that both CPMV RNA and (to a lesser extent) TMV RNA contain cryptic initiation sites that can be activated by fragmentation. It seems likely that most of these sites are never used in vivo (although the 30 000 M_r TMV product is probably a genuine viral protein). The results obtained with the two viruses differ in that the great majority of the products made from fragmented TMV RNA originate at the normal site (at the 5′-end of intact RNA), whereas with fragmented CPMV RNA the 'cryptic' sites seem to be preferred over the normal one. This difference may be explained, at least in part, by the presence of a cap on TMV RNA, which presumably gives a strong competitive advantage to the nearest initiation site [19]. For intact CPMV RNA, some other property must be invoked to explain the restriction of initiation to a single site, despite the presence of other potential sites. The latter may simply be masked by secondary structure, as is known to occur with bacteriophage RNAs [20]. Alternatively, binding of ribosomes to an initiation site might involve an obligatory interaction with an adjacent 5′-end, even if this is not capped; however, there is no direct evidence for such a model.

A practical implication of these results is that translation of degraded RNA may yield, besides 'early quitters', other products arising from initiation at spurious sites, and clearly caution is required in ascribing a physiological role to proteins discovered in this way. The phenomenon may be more marked with uncapped RNAs, but it should be stressed that translation of uncapped RNA fragments can occur under perfectly normal ionic conditions (100 mM added KCl, about 125 mM total monovalent cations) which are optimal for translation of globin and other capped RNAs.

Similar effects are to be expected in ribosome binding experiments. Thus brief micrococcal nuclease treatment of TMV RNA greatly stimulates its binding to ribosomes, but this extra binding is not to the same site as in intact RNA, since binding of the second amino acid, alanine [21], is actually decreased [22]. Artefactual initiation may also occur on the endogenous fragments of globin mRNA that remain after nuclease treatment of the reticulocyte lysate.

Acknowledgements

I thank Drs R. J. Jackson and T. Hunt for advice and many helpful discussions. During the course of this work I was the recipient of a Medical Research Council Research Studentship, and the work was supported by grants from the MRC and CRC to Dr R. J. Jackson.

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