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A short form of the tick-borne encephalitis virus NS3 protein

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Using monoclonal antibodies to the tick-borne encephalitis virus (TBE) nonstructural protein NS3 two forms of this protein were revealed in TBE-infected mammalian cells: a full-length form (69 kDa) and a short form (49 kDa) which has not been observed before and was called NS3′. Recombinant plasmids were constructed and various fragments of the TBE NS3 gene were expressed in rabbit reticulocyte lysate. By analyzing immune precipitates of ^{35}S-labeled translation products, we could monitor and localize internal cleavage of NS3, due to which the NS3′ protein was generated.

Flavivirus; Nonstructural protein; Cleavage

1. INTRODUCTION

Tick-borne encephalitis virus (TBE) belongs to *Flaviviridae*, a family of positive-stranded RNA viruses whose genomes contain a single long open reading frame that is translated into a polyprotein. Co- and posttranslational processing of the polyprotein by viral and host cell proteinases is thought to produce 10 individual proteins, namely, C, prM(M), E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [1]. In this work we found in TBE-infected cells an additional TBE NS3 gene product with molecular weight of 49 kDa which was called NS3′. It is, presumably, a result of an unknown internal processing event in the full-length NS3 protein. We made experiments on the in vitro translation of various fragments of the TBE NS3 gene to clarify the mechanism of the NS3′ protein formation.

2. MATERIALS AND METHODS

2.1. *Immunoblotting of cell preparations*

Suspensions of TBE-infected and noninfected pig embryo kidney cells and human kidney (RH) cells prepared according to [2] were kindly donated by N.A. Belyavskaya (Institute of Natural-Foci Infections, Omsk, USSR) and A.S. Karavanov (Institute of Poliomyelitis, Moscow Region, USSR). Cell probes were subjected to immunoblotting assay [3] using monoclonal antibodies (MoAb) 18B2 [4].

2.2. *Construction of recombinant plasmids*

Standard methods for in vitro DNA manipulations were essentially as described [5]. The full-length TBE NS3 gene was cut from a pUC19NS3 plasmid [4] by *Eco*RI and *Bam*HI restrictases and recloned in a pGEM2 vector [6]. The resulting plasmid pGEM2-NS3 carries the TBE NS3 gene, suitable for in vitro expression. To obtain the deletion mutant form of the gene, the plasmid pGEM2-NS3 was digested with *Eco*RI and *Sal*I. The removed *Eco*RI/*Sal*I-fragment of the gene was then replaced by a synthetic DNA fragment composed of the oligonucleotides: 5′-AATTCCTAGCAATGAG-3′ and 5′-TCGACTCATTGCTAGG-3′. The resulting pGEM2-NS3* plasmid was selected.

2.3. *In vitro transcription and translation*

Plasmid DNAs were linearized with appropriate restrictases. Capped mRNAs were synthesized with SP6 RNA-polymerase using kits for in vitro transcription (Biopol, Moscow, USSR). Preparation of rabbit reticulocyte lysate, in vitro translation and immunoprecipitation of ^{35}S-labeled translation products with TBE hyperimmune mouse ascitic fluid (a kind gift of A.S. Karavanov) and protein A-Sepharose (Pharmacia, Sweden) were conducted essentially according to [7]. Precipitated proteins were analyzed by electrophoresis in 7.5–25% PAAG [8], gels were fluorographed.

3. RESULTS AND DISCUSSION

Recently we have produced MoAb 18B2 to the TBE NS3 protein and revealed its specificity to the protein region localized between 174 and 236 amino acid residues (Ser^{1664}–Met^{1726} of TBE polyprotein [9]) [4]. Using MoAb 18B2, we examined proteins of TBE-infected pig embryo kidney cells (at different times postinfection) and RH cells (48 h postinfection) by immunoblotting. Along with a full-length NS3 protein (69 kDa) we found an additional NS3 gene product of lower molecular weight (49 kDa) undescribed for flaviviruses which was named NS3′ (Fig. 1).

To elucidate a mechanism of NS3′ protein formation in the infected mammalian cells, we carried out experiments on the in vitro expression of NS3 gene and its

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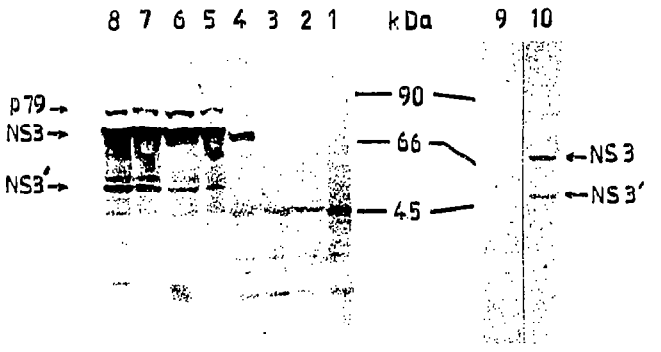

Fig. 1. Immunoblotting of cell preparations conducted using MoAb 18B2. Noninfected (lane 1) and TBE-infected pig embryo kidney cells 2, 4, 8, 16, 24, 45 and 72 h postinfection (lanes 2–8), separation in 10% SDS-PAAG. The p79 protein is likely to be some precursor of NS3. Noninfected (lane 9) and TBE-infected RH cells 48 h postinfection (lane 10), in 7.5–25% gradient SDS-PAAG. Positions of molecular weight markers are indicated.

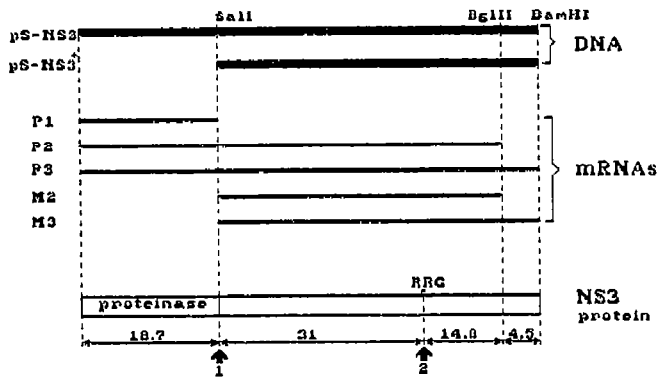

Fig. 2. Scheme of mRNAs produced from pGEM2-NS3 and pGEM2-NS3* plasmid templates. The dashed vertical lines show mRNAs termini projections to NS3 protein. Molecular weights of the NS3 protein fragments are indicated (in kDa). The dot denotes localization of the NS3 protein protease recognition site. Positions 1 and 2 are denoted by vertical arrowheads (see Results and Discussion).

deletion mutants. For this purpose the recombinant plasmids pGEM2-NS3 and pGEM2-NS3* were constructed. The first plasmid contains full-length TBE NS3 gene. Recently it has been predicted [10,11] and demonstrated for a few flaviviruses [12] that 180 N-terminal amino acids of NS3 constitute a protease domain responsible for several cleavages in the course of viral polyprotein processing. The plasmid pGEM2-NS3* contains a mutant form of TBE NS3 gene in which the region, encoding the first 174 amino acids of the protein (putative protease domain), is deleted.

According to the scheme (Fig. 2) those plasmids were linearized and the corresponding mRNAs were synthesized by SP6 RNA-polymerase. The mRNAs were then translated in rabbit reticulocyte lysate. Translation products were immunoprecipitated and electrophoresed in a 7.5–25% linear gradient PAAG.

Similarly to the TBE-infected cells, translation of the full-length NS3-mRNA (P3) yields two products (Fig. 3) of 69 and 49 kDa (NS3 and presumably NS3'). There seem to be three possible reasons for the 49 kDa product formation: (i) premature termination of mRNA translation (at position 2; see the scheme, Fig. 2); (ii) reinitiation of the translation from one of the intermediate Met-codons (at position 1; Fig. 2); (iii) proteolytic cleavage of the full-length NS3 (at one of the two possible positions). If the hypothesis (i) were correct, the translation of the M2 and M3 mRNAs would result in both the full-length products and a protein with molecular weight of 31 kDa. However, only full-length polypeptides were observed under M2 and M3 translation (46 and 50 kDa, respectively; Fig. 3). If the hypothesis (ii) were true, an additional P2 translation product with a lower molecular weight (46 kDa) than in the P3 case (49 kDa) would be synthesized along with the full-length protein (65 kDa). Nevertheless for both P2 and P3 short products were the same (Fig. 3), which also excludes the possibility of NS3 posttranslational cleavage at position 1 (see Fig. 2). Thus the only possible reason for the 49 kDa product formation may be cleavage of the NS3 protein at position 2 (Fig. 2). N-Terminal fragment (putative protease domain) of the protein seems to be involved in this cleavage, because the M2 and M3 translation products are not cleaved.

The results obtained give us a ground to propose that the 49 kDa product observed in vitro and NS3' found in TBE-infected cells should both be formed by the same mechanism due to autocatalytical cleavage of

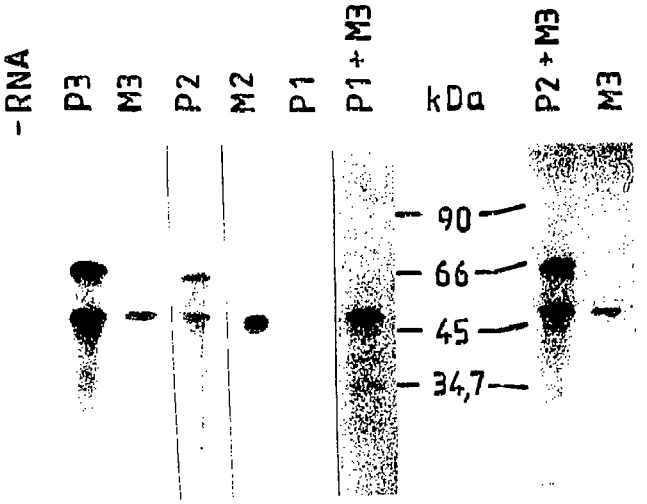

Fig. 3. Expression of the TBE NS3 gene fragments in vitro. Autoradiogram of an SDS-PAAG displaying immunoprecipitated protein patterns from in vitro translation mixtures programmed with mRNAs indicated above each lane. Designations of mRNAs, see in Fig. 2. In the -RNA lane, no mRNA was added. Positions of molecular weight markers are indicated.

NS3. The NS3 protease domain is likely to be required for this cleavage. It occurs presumably at the internal RRG site (1948–1950 amino acids of TBE polyprotein), which is conservative at least for several flaviviruses [9]. To prove the mechanism of NS3′ protein formation, site-directed mutagenesis experiments are being carried out.

If our assumption is true, it should be interesting to elucidate whether viral protease acts at the indicated site in *cys* or *trans*. For this purpose P1 and M3, or P2 and M3 mRNAs were translated simultaneously (Fig. 3). M3 product in those cases may serve as target for a *trans*-acting protease. However, no products of M3 processing were observed, suggesting that cleavage of NS3 occurred only in *cys*. P1 product (19 kDa) is faintly visible in the P1 and P1+M3 lanes upon long exposure only, presumably due to its low radioactivity or to low amount of antibodies in the TBE hyperimmune mouse ascitic fluid specific to this part of the NS3 protein.

As reported recently, under in vivo expression of dengue NS3 gene trace amounts of a 50 kDa additional gene expression product were observed [12]. From our point of view, this protein may be dengue NS3′. Formation of a 50 kDa product was facilitated by NS2B when dengue NS2B-NS3 gene block was expressed. In TBE-infected pig embryo kidney cells the quantity of NS3′ protein was not so large as that observed in vitro (compare Fig. 1 with Fig. 3, lane P3). In addition it is important to note that the efficiency of internal TBE NS3 cleavage was not the same in different rabbit reticulocyte lysate preparations. There was one lysate preparation in which NS3′ was not formed at all (not shown).

Summing up, both viral and cellular factors may be involved in the regulation of at least NS3 internal cleavage in the course of viral polyprotein processing.

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