SUPPLEMENTARY MATERIAL

DISCUSSION

Another issue that should be addressed by future approaches concerns the role of the remaining members of the NFAR group. For example, NFAR-2 might substitute at a certain stage for NF90/NFAR-1 and thus modulate the activity of the protein complex by virtue of its diverging C-terminus. Such a scenario may be indicated by studies of Satoh et al. (1999) showing that NF45 forms a complex with NF90 but also with an NF90-related protein of 110 kDa, which is most probably NFAR-2.

Concerning the functional role of RHA in the cell it was, for example, found that in mammals, the enzyme interacts with RNA polymerase II and the transcriptional co-activator CBP/p300 to stimulate transcription (Nakajima et al., 1997).

The fact that the poliovirus 5’terminus folds into a structure, which, similar to the hairpin Ia in the BVDV genome, modulates translation and RNA replication, was originally shown by Simoes et al., 1991.

MATERIALS AND METHODS

Construction of recombinant plasmids. Restriction and cloning procedures were performed following standard protocols. Restriction and modifying enzymes were purchased from Biolabs (Schwalbach, Germany), Pharmacia (Freiburg, Germany), MBI Fermentas (St. Leon-Rot, Germany), and Roche Diagnostics (Mannheim, Germany), respectively. Oligonucleotides for cloning and
sequencing, the latter 5’IRD71-labelled, were obtained from MWG Biotech (Ebersbach, Germany).

The plasmids to generate the BVDV DI9c replicon RNA (pA/DI9/877) and the 3’NTR transcript (p3’BVDV or pPvu) were described by Meyers et al., 1996 and Yu et al., 1999. The 3’NTR transcript contained the 3’NTR as well as a small part of the 3’-coding region of NS5B. To generate the plasmids for the transcripts encoding the UGA box elements, Oligonucleotides bv2UGAs (5’-GGTTGGGGTACCTCCGGCCTTGTATATATTGTATATAATCTGTATTTGTATATATA TGTTTTAAATTTAGCTTAAGACTAGTC-3’) and oligonucleotide bv2UGAas (5’-GACTAGTCTTAAGCTAAATTTAAACATAATATATACAGATTTATATACATATAT ACAAGGCGGAGTGGTACCCCAACC-3’) were annealed and inserted into the vector pGEMT (Promega). The resulting plasmid was digested with the enzymes KpnI and SpeI (the sites of which were introduced via the applied oligonucleotides; see underlining above) and the digestion product inserted into the pBluescript KS (+) vector (Stratagene) cleaved with the same enzymes. The final plasmid, p2UGA, which encodes nucleotides 10-63 (the 5’UGA box and the UGApapos.cons.box of the 3’V region of the DI9c 3’NTR) in the numbering scheme of Fig. 1B, was linearized with SpeI and transcribed with T3 RNA polymerase (as depicted in Fig. 3C). In the same way, oligonucleotides bvUGAs (5’-GGGTTACCTGTATATATTATGTTAAATTAGGAATTC-3’) and bvUGAas (5’-GGAATTCCCTAAATTTAACCATAATATACAGGTACCCC-3’) were annealed and inserted into pGEMT. The resulting plasmid was digested with KpnI and EcoRI and the fragment introduced into pBluescript KS (+) cut with the same enzymes. Consequently, this plasmid, pUGA, encoded mainly the UGApapos.cons.box of the BVDV DI9c 3’NTR, i.e. nt 40-63 in the nomenclature of Fig. 1B. EcoRI was utilized for linearization of this plasmid to be used as a template for transcription with T3 polymerase (Fig. 3C). To obtain the most frequently applied non-related RNA probe, pBluescript KS (+) was linearized with NaeI and transcribed with T3 polymerase. The different BVDV 5’NTR
probes were obtained by T7 RNA polymerase transcription of differently linearized (BstEII, PstI, EagI and XhoI) BVDV DI9c template DNAs. The EagI site was introduced by Quickchange (Promega) in such a way that the enzyme linearized at position 290 of the BVDV cDNA. The generation of mutant 4 that affected the formation of the stem of hairpin Ia was described in detail by Yu et al. 2000. The genes of the different members of the NFAR family, i.e. NF90/NFAR-1, NFAR-2, NF45 were cloned by PCR from a HeLa cDNA library (ClonTech) and ligated into the multiple cloning sites of pCITE and pCDNA vectors, respectively, using appropriate oligonucleotides primers. The human RHA cDNA clone was a gift from S. Zhang (IMB, Jena, Germany). RHA and the gene of luciferase were cloned in the same way as the NFAR members (details of the cloning and transcription procedures will be provided on request).

The plasmid pSP6(A) was used to generate polyadenylated RNA for pull-down assays. For this purpose the PstI-XmaI fragment of the BVDV DI9c cDNA containing the entire BVDV 3’NTR was cloned into corresponding sites of pSP6(A) to give rise to pSP6(A)3’BVDV. To obtain the plasmid pSP6(A)BKS for the control RNA, the PvuII-XmaI fragment of Bluescript KS(+) was cloned into the HincII and XmaI sites of pSP6(A). In both cases, EcoRI site was used for linearization.

**Preparation of cytoplasmic extracts.** Cells were collected by centrifugation and washed in PBS. The pellet was suspended in lysis buffer (50mM Tris/HCl pH 8.0, 100mM NaCl, 5mM MgCl2, 0.5% NP40, 1mM PMSF) and the nuclei removed by centrifugation (2 min, 1000g).

**Probing of the RNA secondary structure.** The procedures and materials to determine the secondary structure of RNA transcripts of the BVDV 3’NTR were described in detail by Stern et al., 1988, Black and Pinto, 1989 and Yu et al., 1999. Computer predictions of the RNA secondary structure
were performed with the mfold program (http://www.bioinfo.rpi.edu/applications/mfold/rna)

**Transcription, purification, and translation of RNA.** All procedures were described by Behrens et al., 1998. In vitro translation was carried out with reticulocyte lysate (Promega) and \[^{35}\text{S}\] methionine (Amersham) using standard protocols.

**Transfection of RNA.** Viral RNA transcripts and siRNA oligonucleotides were transfected by electroporation using protocols that were previously described by Behrens et al., 1998 and Yu et al. 1999, 2000 (for further details, see also chapter “RNAi”). The transfection efficiency of the viral RNAs was determined by immunofluorescence staining of the NS3 protein, the synthesis of which is only detectable in association with viral replication (Behrens et al., 1998). It was commonly in the range of 80-90% of the surviving cells. The same efficiency of transfection was obtained with siRNA oligonucleotides. In this case, the transfection efficiency was determined by introducing siRNAs directed against the mRNA of GFP (green fluorescent protein) into a cell-line that expressed GFP persistently (data not shown).

**Western blots and antibodies.** Western blots were performed following standard procedures. The applied NF90, NF45 and RHA antisera were described previously (Kao et al., 1994; Zhang and Grosse, 1994).

**Protein sequence analysis.** The sequencing of peptides was performed by the protein sequencing facility of the ZMBH in Heidelberg (Germany).
**RNase Protection Assay (RPA).** The RPA protocol to monitor and to quantify BVDV RNA replication was described previously (Behrens et al., 1998).

**Cell viability assays.** Trypan blue exclusion analysis was performed using a standard protocol. The annexin V test was done according to the guidelines of the manufacturer (BD Biosciences).

**REFERENCES**

**Cited reviews**

**References cited in the supplementary Discussion**
References cited in the supplementary figure legend


SUPPLEMENTARY FIGURE

FIGURE LEGEND

Supplementary Figure. Experimental probing of the BVDV 3’UTR RNA secondary structure.

(A) Structure probing with the single-strand specific chemicals DMS (dimethyl sulfate) and CMCT (1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate). The modification procedures were performed using the protocols, the analogous BVDV DI9c 3’UTR transcript and primer for reverse transcription as described by Yu et al., 1999. DMS was applied as an A and C-specific agent and CMCT as a U and G-specific agent (Stern et al., 1988; Black and Pinto, 1988); however, a high number of G residues was poorly or not modified by CMCT (see also summary in Fig. 7A). The figure shows autoradiographs of the lower (left) and upper (right) part of the same analytical gel (TBE, 7M urea, 7.5% acrylamide) at different exposures. The reverse transcription pattern of the wild type DI9c 3’UTR transcript was compared side-by-side with that of the mutant RNA transcript containing six point mutations in the 5’-terminal UGA box elements (see text and Fig. 7A). (-) without treatment; (DMS) after treatment with 1 μl DMS/μg of RNA; (CMCT) after treatment with 210 μg CMCT/μg RNA. The regions forming the suggested stem and loop of SLstop and the different hairpin structure formed by the mutant RNA (Fig. 7A) are indicated on the right and
left, respectively. The positions of certain nucleotides are marked for orientation. (B) Enzymatic probing of the wild-type BVDV DI9c 3’UTR secondary structure with single-strand specific nucleases. The digestions were performed under denaturing conditions at 55° (left) and 0°C (right) essentially as described by Yu et al., 1999 using 5’-end labeled BVDV DI9c 3’UTR transcript. (T1) RNase T1, G specific; (U2) RNase U2 (A specific); (PhyM) RNase PhyM, U and A specific; (BC) RNase of Bacillus Cereus, U and C specific, cuts also after A; (OH) RNA ladder obtained by limited alkaline hydrolysis; (-) untreated. The numbers on the left represent the positions of G-residues in the RNA transcript. The regions forming the structural features of the BVDV 3’UTR are indicated on the right.

As summarized in Fig. 1B, certain residues in the SL\textsubscript{stop} stem (e.g. A29-31) were found to be partially accessible to chemicals and RNases. The non-specific hydrolysis of the BC nuclease initiating at nucleotide 33 was not considered in the model.