

Generation of Stable mRNA Fragments and Translation of N-Truncated Proteins Induced by Antisense Oligodeoxynucleotides

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Summary

Binding of phosphorothioate-modified antisense oligodeoxynucleotides (AS ODNs) to target mRNAs is commonly thought to mediate RNA degradation or block of translation. Here we demonstrate cleavage of target mRNAs within the AS ODN binding region with subsequent degradation of the 5' but not the 3' cleavage product. Some, if not all, 3' mRNA fragments lacked a 5' cap structure, whereas their poly(A) tail length remained unchanged. Furthermore, they were efficiently translated into N-terminally truncated proteins as demonstrated in three settings: production of shortened hepadnaviral surface proteins, alteration of the subcellular localization of a fluorescent protein, and shift of the transcription factor C/EBP α isoform expression levels. Thus, AS treatment may result in the synthesis of N-truncated proteins with biologically relevant effects.

Introduction

According to current concepts, mRNAs can form closed loops by the interaction of their 5' and 3' ends with associated factors promoting initiation of translation and stability (Gallie, 1998). mRNA degradation is then thought to occur after disruption of this functional messenger ribonucleoprotein (mRNP) particle. At least three types of such events have been determined: poly(A) tail shortening, arrest of translation at a premature non-sense codon (mRNA surveillance), and endonucleolytic cleavage (Beelman and Parker 1995; Hentze and Kulozik, 1999). Steps subsequent to poly(A) tail shortening or premature translational termination lead either to the removal of the 5' cap structure followed by 5'-3' exonucleolytic digestion or to 3'-5' decay of the mRNA body

(Jacobson and Peltz, 1996). mRNA fragments generated by endonucleolytic cleavage are most likely removed by exonucleolytic decay as well, but these events have not been characterized in detail.

An alternative, clinically important way to induce such endonucleolytic cleavage of a target mRNA would be the application of antisense oligodeoxynucleotides (AS ODNs). AS ODNs are known to trigger pleiotropic effects, such as translation arrest, intracellular signaling pathway activation, and CpG-mediated B cell stimulation. The most effective mechanism, however, is attributed to ribonuclease H (RNase H)-mediated target-mRNA degradation (Crooke, 2000). This sequence-specific antisense mechanism is based on the hybridization of the oligonucleotide to a complementary region in the target message. Oligonucleotides possessing anionic internucleoside linkages, either unmodified phosphate diesters or sulfur-modified phosphorothioates, form heteroduplexes that are substrates for the ubiquitous RNase H (Dagle and Weeks, 2000). RNase H cleaves the mRNA component of the heteroduplex. It is generally assumed that the two resulting mRNA cleavage fragments are rapidly degraded in the cell, thus rendering the message permanently untranslatable.

It is now tempting to integrate this RNase H-mediated endonucleolysis into the above-mentioned concepts of mRNA degradation. From a theoretical point of view, two fragments result from this antisense-mediated endonucleolysis: a 5' fragment possessing a cap structure and lacking a poly(A) tail and a 3' fragment possessing a poly(A) tail and lacking a cap structure. The lack of the poly(A) tail of the 5' fragment is thought to lead to subsequent 3'-5' exonucleolytic decay and/or 5'-3' exonucleolysis if decapping occurs. The integrity of the poly(A) tail of the 3' fragment is thought to be unchanged, whereas it may be a substrate for 5'-3' exonucleolysis. There is increasing knowledge about the central role of the poly(A) tail and its associated factors in the cytoplasmic fate of an mRNA (Sachs et al., 1997; Deo et al., 1999). Formation of a functional mRNP via a closed loop enables cap-dependent and -independent translation and protects against exonucleolytic degradation. Therefore, the existence of stable 3' cleavage products may be possible. Indeed, AS ODN-mediated RNase H cleavage products have been reported in different settings, including liver specimens from rats treated with AS ODNs in vivo, *Xenopus* oocytes, chinese hamster ovary cells, human leukemia, and H9 cells (Baba et al., 2000; Shuttleworth and Colman, 1988; Ho et al. 1999; Giles et al., 1995; Veal et al., 1998). However, the fate of these fragments in terms of RNA stability and their mechanisms of degradation are largely unknown and deserve further investigation. We now report the existence of stable 3' mRNA fragments mediated by phosphorothioate-modified AS ODNs in different hepatoma cell lines including the characteristics of their 5' and 3' termini. We further demonstrate that these truncated mRNAs are efficiently translated into N-truncated proteins.

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Results

Generation of Stable 3' mRNA Fragments by Antisense ODNs Directed against DHBV PreS/S mRNA

We previously evaluated AS ODNs as therapy against hepatitis B virus (HBV) in the duck HBV (DHBV) model (Offensperger et al., 1993). It was shown that an AS ODN directed against the start of the preS region resulted in a dramatic antiviral effect in primary hepatocytes and in DHBV-infected Pekin ducks. Here, we further investigated the molecular mechanisms of AS action in avian and human hepatoma cells cotransfected with a replication-competent DHBV construct and phosphorothioate-modified, 18-mer antisense ODNs. The DHBV genome consists of a partially double-stranded, circular DNA molecule of 3021 bp (Sprengel and Will, 1988). Two major transcripts of the DHBV genome are the pregenomic (pg) mRNA (nt 2530–3021/1–2796), which codes for the viral reverse transcriptase/DNA polymerase and the viral core proteins, and the subgenomic (sg) mRNA (nt 740–2796), which codes for the surface (preS/S and S) proteins. As illustrated in Figure 1A, the AS ODN DHBV795 targets simultaneously the first AUG on the sg mRNA and an internal AUG of the pg mRNA. Northern blot analysis of cotransfected avian hepatoma cells revealed that the target mRNAs were not completely degraded. Instead, a distinct band slightly smaller than sg mRNA was specifically generated by AS treatment (Figure 1B). This band was constantly detected over a period of 4 days after transfection. Hybridization of the Northern blot with the probes depicted in Figure 1A indicated that the AS ODN-mediated cleavage of both target mRNAs with subsequent degradation of the 5' but not the 3' fragment. Complete degradation of the 5' fragment was confirmed by primer extension analysis (data not shown). A similar 3' cleavage fragment was observed after cotransfection with AS ODN DHBV795 in human hepatoma cells as shown in Figure 1C. Furthermore, transfections with an AS ODN directed against the third AUG (AS DHBV874) resulted in a slightly smaller 3' cleavage fragment (Figure 1C, lane 4).

Characterization of the Termini of the 3' mRNA Fragment

The stability and degradation of mRNAs are commonly thought to be determined by their 5' cap structures and 3' poly(A) tails. We therefore investigated the termini of the 3' cleavage fragments in more detail.

The existence of a 5' cap structure can be experimentally examined by immunoprecipitation. We applied this technique using the cap-specific antibody H20 (Bochnig et al., 1987) in pilot experiments, hypothesizing that capped uncleaved DHBV mRNA would be immunoprecipitated, whereas uncapped cleaved fragments would not. Unfortunately, we were not able to precipitate sufficient quantities of capped mRNA species from total cellular RNA preparations (either DHBV or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control) for Northern hybridization. As the antibody H20 is directed against virtually all capped cellular messenger and small nuclear RNAs, this technique was not useful because we had to use unconcentrated total cellular RNA preparations.

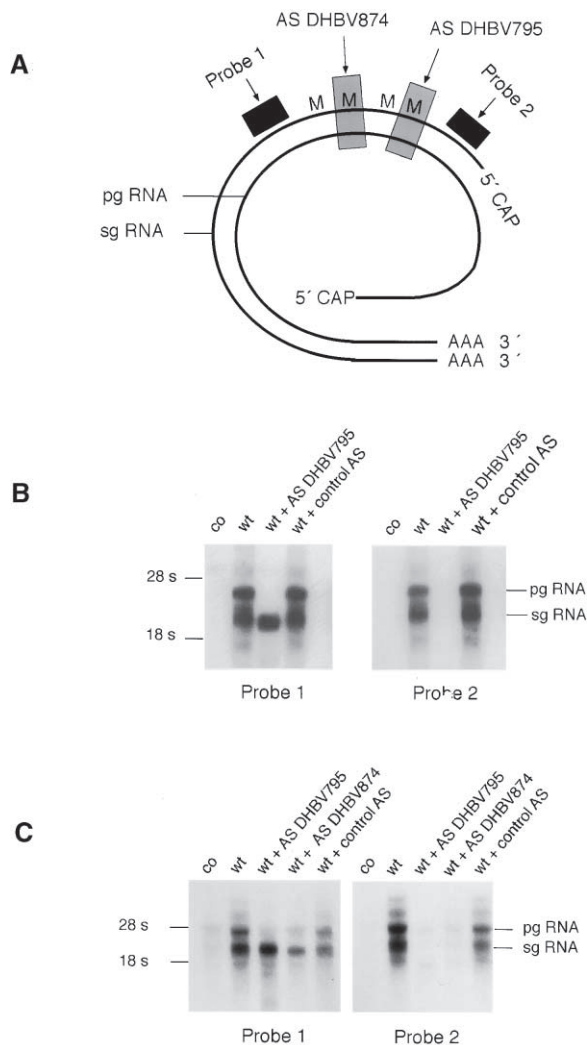


Figure 1. AS ODNs Mediate Cleavage but Not Complete Degradation of the Target mRNA

(A) The DHBV pregenomic (pg) and subgenomic (sg) mRNAs overlap as indicated and the AS ODN and probe binding sites on the mRNAs are illustrated.

(B) Northern blot analysis with oligo hybridization of viral RNA isolated from cotransfected avian hepatoma cells. The pg mRNA is no longer detectable upon cotransfection of AS ODN DHBV795 while a distinct band slightly smaller than sg mRNA was generated. Co, nontransfected cells; wt, DHBV transfected cells.

(C) Similar results were observed in human HuH-7 hepatoma cells using AS ODNs DHBV795 and DHBV874, even if the AS ODN-mediated cleavage by the latter AS ODN was not complete. Equal amounts of RNA were loaded.

If some or all 3' cleavage fragments were uncapped, they should be efficiently ligated using T4 RNA ligase. This reaction requires a monophosphorylated 5' terminus and is prevented by the cap structure. Hence, efficient ligation should only occur in uncapped RNAs. To address this question, a strategy illustrated in Figure 2A was performed (modified from Couttet et al., 1997). First, we circularized the mRNA using T4 RNA ligase. In the next step, cDNA synthesis across the ligated termini was made using primer 912 situated upstream to the cleavage sites and avian myeloblastosis virus reverse

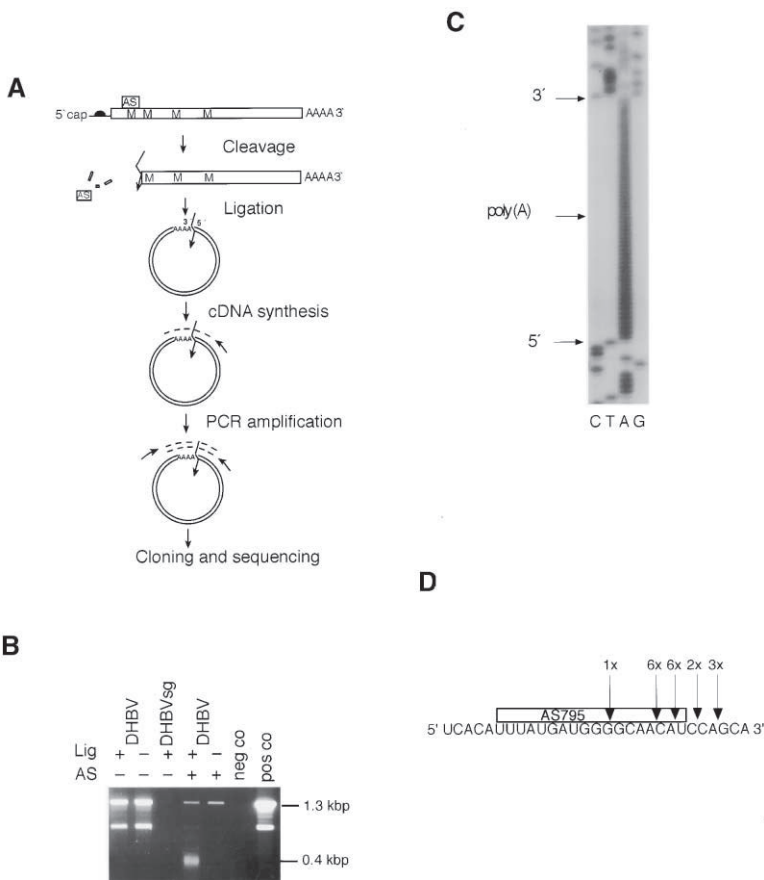


Figure 2. Capping Status of the mRNA 3' Cleavage Fragments and Determination of RNase H-Mediated Cleavage Sites

(A) mRNA 3' cleavage fragments were examined by ligation and subsequent RT-PCR (see text for detailed description).

(B) PCR using primers 860- and 2665+ resulted in a 0.4 kbp fragment in uncapped ligated mRNAs (lane 4). Neg co, water; pos co, DHBV expression plasmid. 1.3 and 0.8 kbp fragments were due to amplification from the pg mRNA (lanes 1 and 2) and/or expression plasmid (lane 7) but not the sg mRNA (lane 3) and did not necessitate the ligation step.

(C) Sequence analysis of cloned PCR products. A representative sequence gel of one clone showing the junction of the 5' end of the cleaved 3' fragment to the poly(A) tail is shown.

(D) Schematic presentation of the AS ODN-mediated cleavage sites indicated by arrows.

transcriptase. This copy of the poly(A) tail and its surrounding sequences were amplified in the third step by using Taq DNA polymerase and two primers that hybridize on both sides of the junction region. Indeed, an expected 0.4 kbp PCR product was detected (Figure 2B, lane 4). Sequence analysis of this PCR product revealed that the 3' cleavage fragments were successfully ligated (Figure 2C). In contrast, uncleaved sg DHBV mRNA was not efficiently ligated and amplified by this procedure. Note that the PCR primers used here also yielded 1.3 and 0.8 kbp fragments that were transcribed from the pg mRNA and/or the DHBV expression plasmid and did not require the ligation step (Figure 2B, lanes 1, 2, and 7). In keeping with this notion, a control plasmid (pDHBVsg) encoding only the sg mRNA did not generate any PCR products (Figure 2B, lane 3).

Sequence analysis of the PCR products revealed furthermore that the 5' ends of the cleavage fragments mapped to the 3' region of the AS ODN/mRNA heteroduplex while the 3' end was intact. The cleavage sites of 18 clones were analyzed and are illustrated in Figure 2D. This cleavage pattern showed that the majority of these events occurred in the distal 3' region on the mRNA in the heteroduplex. The 5'-most cleavage site on the mRNA was 11 nucleotides from the 3' end of the ODN. The 3'-most cleavage site extended 3 residues past the heteroduplex into the single-strand 3' overhang region. No cleavage sites could be observed in the 5' region on the mRNA in the heteroduplex and upstream of the heteroduplex in the single-strand 5' overhang region.

To assess the poly(A) tail length, total cellular RNA from DHBV control (Figure 3, lanes 1–4) and AS ODN treated cells (Figure 3, lanes 5–8) were hybridized to AS ODNs complementary to the DHBV 3' UTR and further cleaved in vitro using RNase H as described (Binder et al., 1994). Two different AS ODNs were used to obtain an internal control of fragment size. Addition of oligo(dT)₁₂₋₁₈ primer to the reactions lead to 3' UTR RNA fragments of 700 and 600 nt, respectively, that lacked a poly(A) tail (Figure 3, lanes 2, 3, 6 and 7), whereas differences of fragment size after omission of oligo(dT)₁₂₋₁₈ directly reflected poly(A) tail length (Figure 3, lanes 4 and 8). The

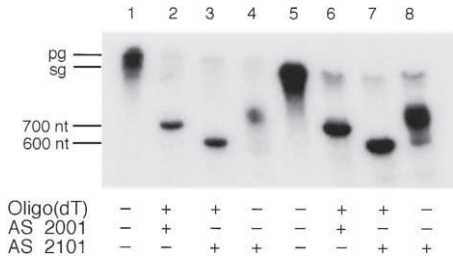


Figure 3. Determination of Poly(A) Tail Length

Poly(A) tail length of 3' cleavage fragments was not significantly different from uncleaved DHBV RNA. RNA from DHBV cells cotransfected without (lanes 1–4) and with (lanes 5–8) AS ODN DHBV795 was hybridized in vitro to the indicated oligos and cleaved by RNase H. This reaction resulted in 3' UTR fragments with the indicated size without (lanes 2, 3, 6, and 7) and with a poly(A) tail (lanes 4, 8).

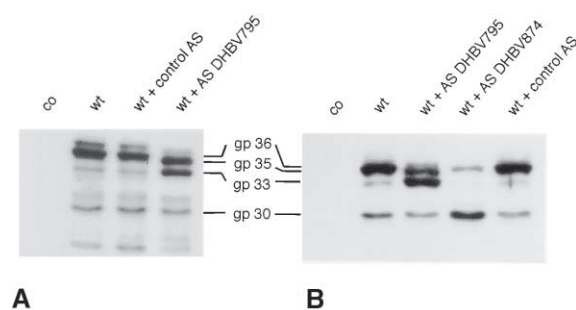


Figure 4. Detection of N-Terminally Truncated PreS/S Proteins in AS ODN Treated Tells

Western blot analysis of viral preS/S proteins isolated from cotransfected avian LMH (A) and human HuH-7 hepatoma cells (B). Co, nontransfected; wt, DHBV transfected cells. Note the relative abundance of gp 35 and gp 33 in cells treated with AS ODN DHBV795 and the abundance of gp 30 in cells treated with AS ODN DHBV874. Equal amounts of protein were loaded.

observed poly(A) tail length distribution did not differ significantly in both populations comprising a peak between 100–200 adenosines with a discrete smear of degradation products.

The 3' mRNA Fragments Are Efficiently Translated into N-Truncated Proteins

Next we analyzed whether the stable 3' mRNA cleavage fragments could still be translated into protein. Western blot analyses of viral preS/S proteins isolated from cotransfected avian and human hepatoma cells were performed (Figure 4). The pattern of viral preS/S proteins in control transfected hepatoma cells without AS ODNs was similar to that obtained during natural infection. The first AUG (nt 801) was predominantly used yielding gp36. Low level translation initiating from the internal AUGs was observed, most likely due to leaky scanning (Fernholz et al., 1993; Kozak, 1986). Initiation of translation at the second AUG (nt 825) lead to gp35, initiation at the third AUG (nt 882) to gp 33, and initiation at the fourth AUG (nt 957) to gp 30. When avian (LMH) or human hepatoma cells (HuH-7 and HepG2) were cotransfected with AS ODN DHBV795, translation of viral preS/S proteins was not abolished. However, instead of the predominant gp36, preS/S proteins with a smaller apparent size than wild-type were detected. The two smaller proteins could be identified as gp35 translated from the next internal AUG located 24 nt downstream of the first AUG and as gp33 translated from the third AUG located 81 nt downstream of the first AUG. This was confirmed by site directed mutagenesis of the respective start codons as well as probing the Western blot with an antibody that recognized aa 9–28 (corresponding nt 825–884) of the preS/S protein (data not shown). Furthermore, AS ODNs directed against different regions of the viral preS/S mRNA ORF (e.g., second and third AUGs) always resulted in translation of N-truncated preS/S proteins starting at the AUG located downstream from the AS target site as shown in Figure 4B for AS ODN DHBV874. This AS ODN is directed against the third AUG and caused a shift of translation initiation to the fourth AUG yielding gp30. The residual expression of gp36 in this

experiment may be due to incomplete cleavage by the AS ODN DHBV874 in HuH-7 cells (see Figure 1C for the corresponding Northern blot).

AS ODN-Mediated Shift of Translation Initiation Results in Relocation of Fluorescence of ECFP-Golgi and Shift of Isoform Expression of C/EBP α

To evaluate whether the generation and translation of stable 3' cleavage fragments would apply to other nonviral mRNA targets, AS ODNs were designed for the ECFP-Golgi (enhanced cyan fluorescent protein) and C/EBP α mRNAs. ECFP-Golgi is a fusion protein consisting of enhanced cyan fluorescent protein (AUG of ECFP: nt 864–866) and a sequence encoding a region of human β 1.4 galactosyltransferase, which contains the membrane anchoring signal peptide that directs the fusion protein to the Golgi apparatus (AUG of the fusion protein: nt 597–599) (Llopis et al., 1998). We hypothesized that an AS ODN-mediated shift of translation initiation to the downstream ECFP-AUG should result in a protein lacking the membrane anchoring protein. Therefore, its fluorescence should be diffusely located throughout the cell. Indeed, AS ODN ECFP-Golgi689 rearranged the intracellular distribution of the fluorescence protein accordingly (Figure 5A). Northern blot analysis revealed the generation of stable 3' cleavage fragments and the degradation of the corresponding 5' fragments (Figure 5B). In cotransfection experiments using a different AS ODN (ECFP-Golgi761) binding 72 nt 3' from AS ODN 689 a slightly smaller 3' cleavage fragment was generated (Figure 5B). Western blot analysis demonstrated the expression of a novel truncated protein (Figure 5C).

As a third example, we examined the mammalian transcription factor C/EBP α as an AS ODN target for the following reasons: various isoforms of the transcription factor C/EBP α are known that arise from unique C/EBP α mRNAs by differential initiation of translation (Calkhoven et al., 2000); full-length C/EBP α (p42) inhibits cell proliferation; and a smaller isoform p30 was shown to be a transcriptional activator lacking the antimitotic activity (Lin et al., 1993). We investigated whether AS ODN could shift isoform expression toward the p30 isoform. Avian hepatoma cells were cotransfected with a plasmid encoding C/EBP α and AS ODN C/EBP α 516. Figure 6 shows that this AS ODN directed against the methionine of p42 shifted the C/EBP α isoform ratio to the isoform p30.

Discussion

Here we report the existence of apparently stable mRNA fragments that were generated by cotransfection with phosphorothioate-modified AS ODN in human and avian hepatoma cells. Such fragments were observed using viral and nonviral mRNAs and various AS ODNs, suggesting that the generation of 3' cleavage fragments is a general phenomenon. Our findings raise the question of how these fragments may resist degradation and be translated in vivo.

Structural features contributing to mRNA stability and translation include the 5' cap structure and the 3' poly(A) tail. N7-methylated guanosine triphosphate (m⁷G) caps are present on all transcripts generated by RNA polymerase II and are involved in pre-mRNA splicing, nucleo-

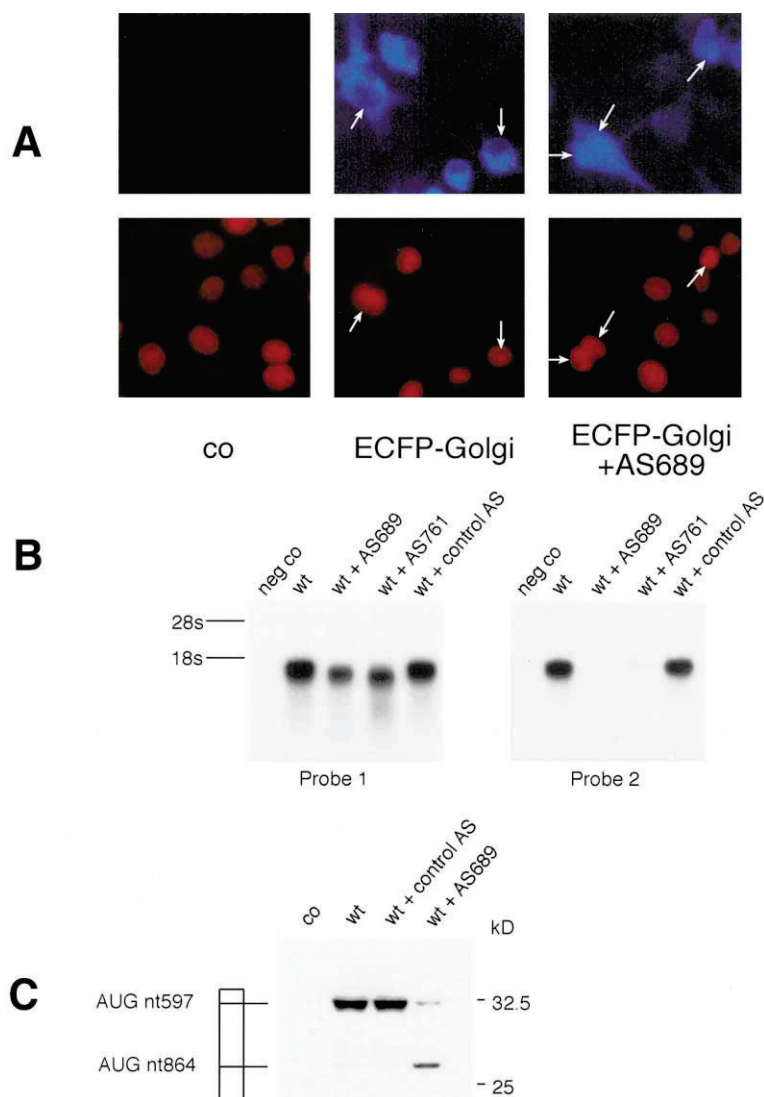


Figure 5. AS ODN-Mediated Translation of Truncated ECFP-Golgi Proteins Rearranges the Subcellular Fluorescence

(A) Fluorescence microscopy of cotransfected avian hepatoma cells. ECFP-Golgi fluorescence is localized only in the cytoplasm and rearranged throughout the cell after AS treatment (upper panels). The cell nuclei were stained by propidium iodide as controls (lower panels). The same time was chosen for exposures.

(B) Again, Northern blot analysis revealed the generation of stable 3' cleavage fragments and the degradation of the 5' fragments in avian hepatoma cells by hybridization with probes located 3' (Probe 1) or 5' (Probe 2) of the AS ODN binding site.

(C) Western blot analysis of Golgi-ECFP proteins isolated from cotransfected avian hepatoma cells. Co, nontransfected; wt, ECFP-Golgi transfected cells. Note that upon AS treatment a smaller protein was detected in cotransfected cells. Schematic representation of the Golgi-ECFP mRNA with the initiation sites indicated in relation to the protein bands on the left. Equal amounts of RNA and protein were loaded.

cytoplasmic transport, mRNA turnover, and translation initiation. Capping of the 5' terminus occurs very early during transcription (Varani, 1997). Since the capped 5' mRNA fragment is removed by AS ODN-mediated cleavage, we anticipated that the stable 3' mRNA cleavage fragments observed here would likely be uncapped.

We used an approach based on RNA ligation and subsequent RT-PCR to assess mRNA capping indirectly (Couttet et al., 1997). This protocol relies on the self-ligation of uncapped 5' monophosphorylated RNA, which is prevented by m⁷G-caps. We have shown the successful ligation of the 3' cleavage fragments by RT-PCR. Specificity and ligation were confirmed by cloning and sequencing. This finding suggests that the mRNA 3' cleavage fragments are uncapped. However, even if ligation-based RT-PCR has previously been shown to be sensitive to the capping status of the mRNAs (Couttet et al., 1997), it is difficult to quantify the amount of capped versus uncapped cleavage fragments in this indirect and exponential assay. Therefore, we cannot rule out the possibility that a fraction of 3' fragments may be recapped after cleavage by an as yet

unknown mechanism enabling cap-dependent stabilization and translation.

An advantage of ligation-based RT-PCR lies in the possibility to exactly determine the AS ODN-mediated cleavage sites. The cleavage sites observed in our experimental setting are in good agreement with recent *in vitro* findings using RNase H1 from *Escherichia coli* (Lima and Crooke, 1997). We therefore conclude that AS ODN-mediated cleavage is sequence specific and is most likely catalyzed by RNase H.

3' Polyadenylation of approximately 250 adenosines is an early event during the synthesis of mRNAs in the nucleus (Wahle and Keller, 1996). The poly(A) tail length is then thought to influence the cytoplasmic half-life and translation efficiency. The poly(A) tail lengths of uncleaved DHBV mRNAs and the 3' cleavage fragments were assessed using RNase H, oligo(dT)₁₂₋₁₈, and primers complementary to the 3' UTR *in vitro* as described (Binder et al., 1994). Our data indicate that poly(A) tail length of 3' cleavage fragments remained unchanged with a peak between 100 and 200 adenosines. Deadenylation of uncapped 3' mRNA fragments may even be

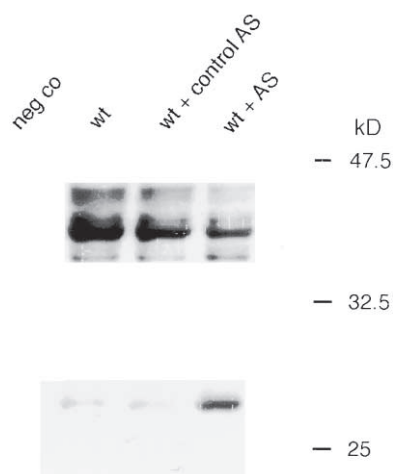


Figure 6. AS ODN Shifts C/EBP α Isoform Expression toward p30
Western blot analysis of C/EBP α isoforms isolated from cotransfected avian hepatoma cells. Neg. co, nontransfected; wt, C/EBP α transfected cells. Note the relative abundance of p30 in treated cells. Equal amounts of protein were loaded.

retarded because deadenylation by a poly(A) specific exoribonuclease (PARN) was recently shown to be stimulated by the m⁷G cap structure (Dehlin et al., 2000; Gao et al., 2000).

Our findings strongly suggest that AS ODN-generated 3' cleavage fragments serve as templates for translation since N-truncated proteins matching the primary structure of the respective 3' cleavage fragments were observed, whereas the full-length mRNA was undetectable following AS treatment. This raises the question of how these 3' fragments are translated and stabilized. Binding of eukaryotic initiation factors of translation like eIF4G to the mRNA and subsequent recruitment of the 40S ribosomal subunit is a critical step in eukaryotic initiation of translation (de Gregorio et al., 1999; Hentze, 1997). eIF4G interacts with both the 5' m⁷G cap via eIF4E and the 3' poly(A) tail via the poly(A) binding protein (PABP) (Morino et al., 2000; Imataka et al., 1998). These factors can either promote translation alone or synergistically (Sachs et al., 1997; de Gregorio, et al. 1998; Preiss and Hentze, 1998). However, the presence of a cap structure is considered to be crucial for translation *in vivo*. An alternative pathway independent of 5' caps and poly(A) tails is translation via internal ribosome entry sites (IRES) (Sachs et al., 1997).

Although our experiments do not allow insights into the precise molecular mechanisms underlying the translational events, one may speculate that the poly(A) tail plays a major role in this setting. If neither a cap structure nor an IRES is available as suggested here, the poly(A) tail may mediate the 40S ribosome subunit recruitment to the mRNA (Sachs et al., 1997). Until now, only polyadenylated viral mRNAs have been shown to be a template for efficient translation independent of a 5' cap or an IRES (Gallie, 1998). In one example, a translational enhancer in the 3' UTR mimicking a cap has been described (Wang et al., 1997). However, similar structures have not been described in the mRNAs examined here.

Translation, stability, and degradation of mRNAs are

tightly coupled processes in mammalian cells. Degradation pathways include deadenylation-dependent decapping and fast 5'-3' exonucleolysis, and 3'-5' exonucleolysis and endonucleolytic cleavage with subsequent exonucleolytic decay (Beelman and Parker, 1995). In yeast, decapping prior to fast exonucleolytic 5'-3' digestion has been described as a major pathway of mRNA decay (Tucker and Parker, 2000). Indirect evidence suggests that this pathway may be active in mouse liver cells as well (Couttet et al., 1997). On the other hand, 3'-5' exonucleolytic decay has been described in mammalian cells (Brewer, 1999). The capped 5' cleavage fragments generated by AS ODN action most likely lack a poly(A) tail. Their rapid degradation would therefore be compatible with both exonucleolysis in a 3'-5' direction and perhaps in a 5'-3' direction if decapping occurs. If 3'-5' decay was the predominant degradative pathway, the poly(A) tail in concert with poly(A) binding proteins would stabilize the 3' cleavage fragments. In addition, binding of translation factors and/or the translational machinery to the 3' cleavage fragments might help to prevent exonucleolytic attack. Several stability determinants other than 5' caps and 3' poly(A) tails have been described (Guhaniyogi and Brewer, 2001), some of them localized in the 3' UTR, and may also contribute to our findings.

In conclusion, our findings suggest the following mechanism of AS ODN action: target mRNAs are cleaved by RNase H into two fragments. The capped 5' fragment lacking a poly(A) tail is rapidly degraded, whereas the uncapped 3' fragment appears to be stable, perhaps due to its poly(A) tail and binding factors. This fragment can then be translated into protein.

These findings have several implications for both cellular biology and drug therapy. (i) We provide a useful model for the study of stability and translational efficiency of probably uncapped mRNAs. Especially the structural prerequisites needed for stability and translation deserve further characterization. (ii) The AS ODN strategy could be used to generate novel polypeptides with desired properties from the endogenous cellular repertoire. As shown here for the transcription factor C/EBP α , promising targets may be proteins that are translated into functionally different isoforms by leaky scanning such as transcription factors or protooncogenes, e.g., C/EBP α , C/EBP β , int-2, and BAG-1 (Kozak, 1986; Cazzola and Skoda, 2000). (iii) Finally, the therapeutic use of AS ODN may have to be reevaluated in the light of our findings. The translation of novel, N-terminally truncated polypeptides with unexpected and biologically relevant effects may cause serious side effects of antisense therapy.

Experimental Procedures

Plasmids

Wild-type DHBV surface proteins were expressed from plasmid pGemDHBV, a replication-competent head-to-tail dimer construct (von Weizsäcker et al., 1995). pDHBVsg is a plasmid encoding only the DHBV sg RNA and was obtained by digestion of pGemDHBV with *DraI* and *DraIII* and insertion of a resulting 2060 bp fragment into the pCDNA3.1 vector (Invitrogen, Groningen, Netherlands).

pECFP-Golgi (Clontech, Heidelberg, Germany, Cat. # 6908-1) encodes a fusion protein consisting of enhanced cyan fluorescent protein and a sequence encoding the N-terminal 81 amino acids of human β 1.4-galactosyltransferase. pCMVex m- α encodes the transcription factor C/EBP α driven by a CMV promoter.

Antisense Oligonucleotides

Phosphorothioate-modified, HPLC-purified ODNs were obtained from Microsynth (Balgach, Switzerland). AS ODN DHBV795: ATGTT GCCCATCATATAA; AS ODN DHBV874: TTGGGATCATTCTCCGG; AS ODN ECFP-Golgi689: GTGACGCCAAGGTGCAGA; AS ODN ECFP-Golgi761: TGCAGCGGTGTGGAGACT; AS ODN C/EBP α 516: GGAG TCGGCCGACTCCAT.

Cell Culture and Transfections

LMH chicken hepatocellular carcinoma cells (Kawaguchi et al. 1987) were cultured in Isocove's modified Dulbecco's medium (GIBCO-BRL, Life Technologies, Karlsruhe, Germany). Human hepatoma cell lines HepG2 and HuH-7 were cultured in DMEM (PAN Biotech, Aidenbach, Germany). Media contained 8% fetal bovine serum (Biochrom, Berlin, Germany), Penicillin (100U/ml), Streptomycin (100 μ g/ml, both GIBCO-BRL), and 1% of a nonessential amino acid stock solution (GIBCO-BRL). Cells were transfected by the calcium phosphate method. In a typical cotransfection experiment, 5 μ g of plasmid and 5 μ g of antisense ODNs were used per 10 cm dish. To exclude the possibility that our results were due to artifacts after calcium phosphate transfection, we performed control transfections of pGemDHBV and AS ODNs 795 and 874 with the nonliposomal lipid Effectene (Qiagen, Hilden, Germany) with comparable results. Cells were harvested two days after transfection.

Northern Blot Analysis

Total cellular RNA was prepared using the RNeasy Mini Kit (Qiagen). Samples were treated with 5 U DNase I (GIBCO-BRL) for 30 min to eliminate plasmid DNA. The RNA was separated on MOPS (morpholinopropanesulfonic acid)-buffered formaldehyde containing 1% agarose gels. RNA was blotted onto Nylon membranes (Hybond N, Amersham Pharmacia Biotech, Freiburg, Germany) by capillary transfer using 1.5 M NaCl, 150 mM sodium citrate. RNA was visualized by hybridization with ^{32} P labeled oligo probes.

RNA Ligation, RT-PCR, Cloning, and Sequencing

5 μ g of total cellular RNA was ligated by 100 U T4 RNA ligase (New England Biolabs, Schwalbach, Germany) with 40 U RNasin (Promega, Mannheim, Germany) for 1 hr at 37°C. RNA was then extracted using the RNeasy kit. During the second step, ligated RNA was reverse transcribed using primer 912- (located downstream from the AS ODN target site, CTGACCATGTCAAAGTCC) and AMV-reverse transcriptase (Superscript II, GIBCO-BRL) for 90 min at 42°C. The cDNA was amplified by PCR using primers 912- or 860- (located upstream from primer 912-, CAATATTCTCCTCCTTC) and 2665+ (located downstream from primer 912-, AGAGCCTTAGC CAATGTGTAT). 30 cycles (1 min 94°C, 1 min 55°C, and 1.5 min 72°C) were performed. The ligated RNA transcript specifically generated a 0.4 kbp PCR product that was excised from the agarose gel and cloned. The cloned PCR products were sequenced with ^{32} P-labeled primers. All enzymatic reactions were performed in the appropriate buffer provided by the manufacturer.

Assessment of Poly(A) Tail Length

Poly(A) tail length was measured as described (Binder et al., 1994). Approximately 5 μ g of total cellular RNA was hybridized to 20 pmol AS ODN 2101 (complementary to DHBV nt 2101–2118) and cleaved with RNase H (5 U, USB, Cleveland, OH) in RNase H-Buffer provided by the manufacturer for 30 min at 37°C. This reaction cleaved DHBV mRNAs 600 nt upstream of the poly(A) tail. Where indicated, the poly(A) tail was removed by including 50 pmol oligo(dT)_{12–18} in the reactions. In another sample, AS ODN 2101 was replaced by AS ODN 2001 complementary to nt 2001–2018. The resulting 700 nt fragment served as an internal size marker. The reaction products were denatured by urea containing 6% PAGE (Sequagel, National Diagnostics, Atlanta, GA), electrophoretically on nylon membranes, and hybridized with a ^{32} P labeled probe complementary to nt 2281–2313.

Western Blot Analysis

For immunoblot analysis, the following three different polyclonal antibodies were used: an antibody against DHBV surface antigen (Lambert et al., 1990), an antibody that recognizes all green fluorescent protein variants including fusion proteins (Clontech, Heidel-

berg, Germany, Cat.# 8367-1), and an antibody against transcription factor C/EBP α (Santa Cruz Biotechnology Inc., Santa Cruz, Cat.# sc-61). Cell extracts were denatured, separated by SDS-PAGE (12%), and electroblotted to PVDF membranes (Immobilon, Millipore, Bedford). After blocking, the membrane was incubated with the primary antibody overnight. Using the enhanced chemiluminescence detection method, the sequential steps were carried out according to the instructions of the manufacturer (Pierce, Rockford, Cat. # 34080).

Fluorescence Microscopy

For fluorescence microscopy, cells were washed with PBS and fixed for 15 min with 2% paraformaldehyde and 0.05% Triton X in PBS. After washing with PBS, the nuclei were stained with 1 μ g/ml propidium iodide and 200 μ g/ml RNase A (Qiagen) in PBS. Photos were taken using the same exposure times using specific fluorescence filters for propidium iodide and ECFP.

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Update

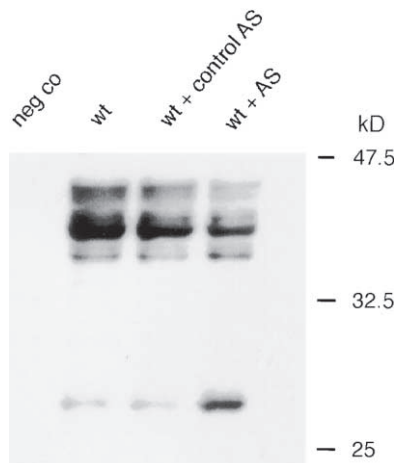
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Erratum

In the October 2001 *Molecular Cell* article by Thoma et al., "Generation of Stable mRNA Fragments and Translation of N-Truncated Proteins Induced by Antisense Oligodeoxynucleotides," (volume 8, 865–872) the data in Figure 6 were printed incorrectly. Parts of the gel were inadvertently omitted due to a technical error. The figure is printed correctly below. We apologize for the error and for any confusion it may have caused.



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