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The Role of the 5' Untranslated Region of Eukaryotic Messenger RNAs in Translation and Its Investigation Using Antisense Technologies

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I. General Aspects of Translation Initiation in Eukaryotes

The expression of genetic information in eukaryotic cells is modulated at different stages by a variety of complex biochemical mechanisms. During the past few years, it has been well established that mRNA translation is an important means of controlling gene expression, and an increasing number of genes have been shown to be regulated at the translational level.

The mRNA translation pathway can be divided into three kinetic phases: initiation, elongation, and termination. The initiation phase includes multiple steps that ultimately result in the recruitment of a translation-competent 80-S ribosome to the initiation codon of an mRNA and the onset of polypeptide chain synthesis. A large set of eukaryotic initiation factors (eIFs) is involved in this process, which mediates the interactions among mRNA, initiator-tRNA, and the ribosomal 40-S and 60-S subunits. The region 5' of the translation initiation codon of an mRNA is called the 5' untranslated region (UTR), 5' noncoding region, or "leader" sequence, and plays an important role in the initiation of protein synthesis.

Once the first peptide bond is formed, the addition of amino-acid residues to the growing polypeptide chain proceeds (elongation). Elongation is promoted by four elongation factors, and includes binding of aminoacyl-tRNAs to the "A-site" of the ribosome, followed by GTP hydrolysis, formation of the peptide bond, and translocation to the "P-site." When the ribosome encounters one of the three termination codons, the newly synthesized polypeptide is cleaved from the peptidyl-tRNA (termination) by a release factor. How the 80-S ribosome dissociates from the mRNA has not yet been precisely defined. The elongation and termination phases of translation are mechanistically better defined than is the initiation step, and the biochemical pathways resemble those of bacteria in most major aspects (for reviews on translation in prokaryotes, see 1 and 2, and in eukaryotes, 3-5). On the other hand, eukaryotic translation initiation differs in many aspects from the situation in bacteria, and, not surprisingly, eukaryotic mRNAs possess structural features different from their bacterial counterparts. During the past few years, biochemical and genetic studies have provided sufficient data on the assembly of the initiation machinery and the function of specific factors to allow mechanistic models for translation initiation to be proposed. However, several important molecular events remain poorly defined, and their elucidation provides a challenge to those working in the field.

This article aims to summarize recent advances in the field of translational control, and to discuss the possibility of applying the powerful antisense technology to investigate some of the unanswered questions, especially those pertaining to the role of the 5' UTR on translation initiation.

Translational regulation is predominantly exerted during the initiation phase, which is considered to be the rate-limiting step (6–8). Two types of translational regulation can be distinguished: *global*, in which the initiation rate of (nearly) all cellular mRNAs is controlled, and *selective*, in which the translation rate of specific mRNAs varies in response to biological stimuli. In most cases of global regulation, control is exerted via the phosphorylation state of certain initiation factors, whereas only a few examples of selective regulation have been characterized well enough to define the underlying molecular events. Interestingly, *cis*-acting regulatory sequences affecting translation initiation have been found not only in the 5' UTRs of selectively regulated mRNAs, but also in the 3' UTRs. Thus, in addition to the protein-encoding open reading frames, both the 5' and 3' UTRs of mRNAs must be considered for their effect on translation.

A. Features of the 5' UTR in Eukaryotic mRNAs That Affect Translation Initiation

1. THE CAP STRUCTURE

All known eukaryotic (nonorganellar) and most viral mRNAs possess a characteristic structure at the 5' end, termed the "cap." The cap is an inverted monomethyl-guanosine nucleoside attached to the 5' end of the mRNA via a 5'–5' triphosphate linkage and is added posttranscriptionally. It has the general composition $m^7G(5')ppp(5')N$, where N represents the first transcribed nucleotide encoded by the corresponding gene. The cap protects pre-mRNAs in the nucleus (9) and mature mRNAs in the cytoplasm (10) from 5'→3' exonucleolytic degradation. It has recently been shown to constitute a positive signal for small nuclear RNA (snRNA) (11) and mRNA (12) transport from the nucleus to the cytoplasm of *Xenopus laevis* oocytes.

The primary feature attributed to the cap structure is the enhancement of translational efficiency (13). The presence of the methyl group on the guanosine ring seems to be necessary for translational activation *in vivo*; a non-methylated cap does not stimulate translation, although it retains its ability to stabilize the mRNA. This phenomenon was observed in mutants of vesicular stomatitis virus defective in cap methylation (14). Additionally, conditions under which the cellular factors that normally interact with the cap are functionally impaired cause a profound reduction in mRNA translation (see Section I,C,3). However, some exceptions have been reported from *in vitro* studies (15). In general, the cell-free translation system derived from wheat germ displays a stronger cap requirement than that from rabbit reticulocytes (16).

It should be noted that the RNA genomes of the animal picornavirus family and of some plant retroviruses are uncapped, but these RNAs are

nevertheless translated very efficiently in virus-infected cells, as well as in some cell extracts, in a cap-independent manner (17, 18). By employing an RNA transfection assay in HeLa cells, it was shown that when a luciferase mRNA was fused at the 5' UTR with 5' noncoding sequences of poliovirus mRNA, the presence of a cap *reduced* the translational efficiency, as compared to that of identical uncapped transcripts (19).

2. LENGTH AND SECONDARY STRUCTURE

Statistically, the 5' UTR of an average vertebrate mRNA varies in length between 20 and 100 nucleotides (20). Very few natural transcripts have a 5' UTR of fewer than 20 nucleotides (20), and synthetic transcripts need a leader longer than this for efficient translation (21). A number of mRNAs, including several viral RNAs, contain remarkably long 5' UTRs in the range of 400–1200 nucleotides. Many cellular transcripts for proto-oncogene products, mitogens, or growth factors fall into this category. A possible reason for this is discussed in Section II,C,2. Long leaders, especially those containing (G+C)-rich sequences, have the potential to form stable hairpin structures which impede translation (22). In contrast, test transcripts with long unstructured leaders are translated efficiently (23).

The rate of initiation is reduced by secondary structures in the 5' UTR in a position-dependent manner (24–26). Secondary structures of moderate stability ($\Delta G = -30$ kcal/mol) in close proximity to the cap (within the first 12 nucleotides) inhibit access of a subset of initiation factors to the cap (24) and the binding of the 40-S ribosomal subunit to the mRNA (27) (see also Section IV,A,2). Additional experiments in cultured monkey COS cells and in cell-free translation extracts revealed that similar hairpins further downstream of the cap can be readily melted by the initiation machinery, whereas structures with Gibbs energies in the range of -50 kcal/mol are resistant (26, 27) and appear to block scanning by the 43-S preinitiation complex (see Section I,C,1). The above conclusions have largely been confirmed by studies in *Saccharomyces cerevisiae*, both *in vivo* and *in vitro* (28–30).

In accordance with these results, positioning of the AUG codon within a moderately stable secondary structure does not negatively affect initiation. Interestingly, normally poor translation of mRNAs with strong secondary structures in the 5' UTR 57 nucleotides downstream of the cap is markedly enhanced in NIH 3T3 cells transfected to overexpress the cap-binding protein eIF-4E (31), indicating the functional role of this factor in recruiting RNA helicase activities to the mRNA.

In contrast to the inhibitory effect of stable secondary structures in the 5' UTR, a hairpin downstream of the initiation codon can exert a positive effect on the selection of the upstream AUG codon by the preinitiation complex, independent of its sequence context (see next paragraph). The hairpin is

thought to slow the migration of the preinitiation complex and thus facilitate the AUG recognition process (32).

3. NUCLEOTIDE CONTEXT OF THE INITIATION CODON

A statistical analysis of the nucleotide sequences that flank the initiation codon AUG in vertebrate mRNAs identified the consensus sequence (A/G)CCAUGG (20). Similar compilations of mRNA sequences derived from *Drosophila melanogaster* (33), yeast (34), and plants (35) revealed variation in the start codon context among different eukaryotic groups. However, in all groups an obvious preference for a purine (A>G) at the -3 position upstream of the AUG was apparent. The conservation of sequences around the AUG start codon suggests a functional significance with regard to translation initiation, an assumption validated by mutational studies (36-38). The sequence of GCC(A/G)CCAUGG emerges from mutational analyses as the optimal nucleotide context for translation initiation in mammalian cells. The purine at position -3 and the G at position +4 appear to be particularly relevant for efficient AUG selection. The molecular basis for this bias remains obscure.

4. UPSTREAM AUGS AND UPSTREAM OPEN READING FRAMES (uORFs)

Several eukaryotic and viral mRNAs contain one or more AUG codons, or even one or more short ORFs in the 5' noncoding region. Due to the low reinitiation frequency of eukaryotic ribosomes, the presence of upstream AUGs generally reduces the translational efficiency of the major ORF. Removal of uORFs or AUGs therefore relieves these inhibitory effects (39), although the possibility that the presence of upstream AUGs can sometimes represent cDNA cloning artifacts must also be considered (22). For the cap-independent translation of some viral RNAs (see Section I,C,3), AUG-rich noncoding sequences do not pose a problem for efficient translation and are, in fact, even required in some instances. uORFs are essential regulatory components of the selective regulation of the yeast GCN4² mRNA, which is discussed in Section II,A.

5. BINDING SITES FOR REGULATORY PROTEINS

The most striking example is the iron-responsive element (IRE), a sequence present in the 5' noncoding regions of ferritin and the erythroid-specific form of 5-aminolevulinic synthase (eALAS) mRNAs. It is responsible for the iron-dependent translational regulation of ferritin and eALAS

² Abbreviations are explained in Section V, pp. 227-228.

mRNAs by interacting with the cytosolic "iron regulatory protein" (IRP) (for details, see Section II, B).

B. The Translation Initiation Pathway

The process of initiation includes the steps that, after selection of a particular mRNA by the translation machinery, lead to the onset of polypeptide synthesis at the appropriate initiation codon. Several eukaryotic initiation factors are involved in the assembly of intermediate complexes and promote mRNA-protein interactions. For details concerning the functions of eIFs, see 5. The initiation pathway can be divided into the following phases.

1. DISSOCIATION OF RIBOSOMAL SUBUNITS

60-S and 40-S ribosomal subunits not engaged in translation have a tendency to associate with one another and form 80-S ribosomes without mRNA. These 80-S ribosomes are inactive in translation and must be separated into 40-S and 60-S subunits for the formation of active initiation complexes to proceed. Three initiation factors affect the equilibrium between associated and dissociated subunits: eIF-1A and eIF-3 interact with the 40-S subunit, and eIF-3A with the 60-S subunit *in vitro*, preventing association (40-42). However, it is not clear whether these factors are sufficient for the dissociation in living cells, or whether additional components are also required.

2. Met-tRNA_i BINDING TO THE 40-S RIBOSOMAL SUBUNIT: FORMATION OF 43-S PREINITIATION COMPLEXES

Initiation factor 2 (eIF-2), GTP, and Met-tRNA_i can form a fairly stable ternary complex. Complexes between the 40-S subunit and this ternary complex (43-S) can be isolated on sucrose gradients, while stable mRNA·40-S complexes cannot, unless Met-tRNA_i is present (43, 44). These findings support the prevailing hypothesis, that the ternary complex binds to the 40-S ribosomal subunit prior to mRNA. However, the possibility that unstable mRNA·40-S intermediates, which have so far escaped detection, are formed in the absence of Met-tRNA_i cannot be ruled out, and a kinetic analysis is required to definitively elucidate the order of assembly.

3. ASSOCIATION OF THE mRNA WITH THE 43-S PREINITIATION COMPLEXES: RECOGNITION OF THE INITIATION CODON

The association of mRNA with the ribosome and the recognition of the AUG codon are aspects of translation initiation that are incompletely understood. Unlike bacterial mRNAs, which possess structural features (Shine-Dalgarno sequences) that facilitate a direct interaction with the ribosome

through hydrogen bonding with the rRNA (for a review on prokaryotic translation initiation, see 45), eukaryotic mRNAs seem to lack comparable recognition elements. Several eukaryotic initiation factors (the eIF-4 polypeptides), which are involved in the interaction of the mRNA with the 43-S preinitiation complex, have been identified and biochemically characterized: eIF-4E (24 kDa) directly binds to cap structures, while eIF-4A (44 kDa) has RNA-unwinding activity, which is ATP-dependent and requires eIF-4B (46). eIF-4A contains the characteristic "DEAD" box RNA helicase motif, and the relevant ATP-binding and catalytic domains have been mapped (47) (see also Section III, D). The cap-binding protein eIF-4E and the eIF-4A/4B helicase can be isolated alone or as parts of the eIF-4F multiprotein complex together with a 220-kDa polypeptide, termed p220, whose function is still unknown. eIF-4E is considered to be a major site of global translational regulation, and its stimulatory activity on translation correlates with the phosphorylation state of the protein, perhaps by stabilizing interactions between eIF-4E and p220 in the eIF-4F complex (48, 49).

Several models, not mutually exclusive, have been proposed for the pathway by which the mRNA and 43-S preinitiation complexes associate. According to one model, which probably accounts for most cellular mRNAs, the 5' cap structure is first recognized by eIF-4F, which facilitates binding of the 43-S complex. Subsequently, secondary structures within the 5' UTR are melted during linear 5'→3' "scanning" until the AUG codon is encountered ("scanning model") (50, 51). An alternative model states that the 43-S complex enters directly at an internal site within the 5' UTR (internal initiation model) (52–54). The two models and their variations are discussed in more detail in Section I, C.

The ultimate purpose of the 43-S preinitiation complex is to reach and identify the initiation codon. A major role in the recognition process is attributed to eIF-2. Genetic studies in *S. cerevisiae* have identified mutations in the α and β subunits of eIF-2 that allow aberrant (AUG) start-codon selection (55, 56). Once the 43-S complex is directed to the initiation codon, with the aid of eIF-2, the anticodon of Met-tRNA interacts directly with the AUG codon by base-pairing (57). As previously mentioned, the context around the AUG is a critical determinant for the recognition, but what makes a context favorable or not is currently unknown in molecular terms.

4. JOINING OF THE RIBOSOMAL SUBUNITS

The association of the 60-S ribosomal subunit with the 43-S complex is stimulated by eIF-5, and requires the positioning of the 40-S subunit, carrying the ternary complex (Met-tRNA_i):(eIF-2):GTP, on the initiation codon of the mRNA. The GTP molecule is hydrolyzed to GDP, and eIF-2:GDP is released together with other bound factors (44, 58). The release of ribosome-

dissociating factors results in the joining of the ribosomal subunits. The eIF-2-GTP binary complex is regenerated by exchange of GDP from the ejected eIF-2-GDP with GTP. This exchange reaction is catalyzed by eIF-2B, also known as guanine nucleotide exchange factor (GEF), since the binding affinity of GDP to eIF-2 is 400 times higher than that of GTP (59).

C. Models for the Association of mRNA with the Ribosome

1. THE SCANNING MODEL

The scanning model postulates that the 43-S preinitiation complex first interacts with the mRNA at or close to the 5' cap structure, moves (scans) linearly in a 3' direction, and stalls as it reaches the initiation codon AUG. Initial evidence supporting this model was obtained when capped reoviral mRNAs were translated *in vitro* in the presence of edeine, an antibiotic that perturbs the AUG recognition process (60). Complexes that sedimented rapidly in glycerol gradients were obtained. Further mapping revealed that these complexes represent multiple 40-S ribosomal subunits distributed all over the mRNA. The apparent interpretation was that the 40-S subunit normally associates with the mRNA at an entry site at the 5' end and moves toward the initiation codon where the 60-S subunit joins. Edeine prevents this step, resulting in aberrant scanning and accumulation of multiple 40-S ribosomes along the mRNA. According to the scanning hypothesis, the 43-S complex moves until it encounters a suitable initiation codon. Thus, if an upstream AUG is inserted in phase with the main reading frame, an N-terminally extended polypeptide is synthesized (61, 62), whereas insertion of an out-of-phase upstream AUG causes the ribosome to translate an unrelated protein (63, 64). When the context of an AUG is unfavorable, it is bypassed by the 43-S complex, which continues scanning until it encounters a "better" AUG, a process called "leaky scanning" (51 and references therein). A simplified scheme for the scanning model is depicted in Fig. 1.

The basic idea of the scanning model has been widely accepted, in spite of the lack of direct kinetic data. The biochemical characterization of initiation factors helped to extend the scanning hypothesis, and modifications of the initial model have been proposed that accommodate the known function of the eIF-4 polypeptides. The first factor that interacts with the mRNA is thought to be the cap-binding protein eIF-4E. Since eIF-4E is found in a substoichiometric ratio with respect to mRNAs and other initiation factors (65), mRNAs compete for binding. This step may therefore represent a selection mechanism for mRNAs to be translated.

It is not clear whether eIF-4E interacts with the cap as a single polypep-

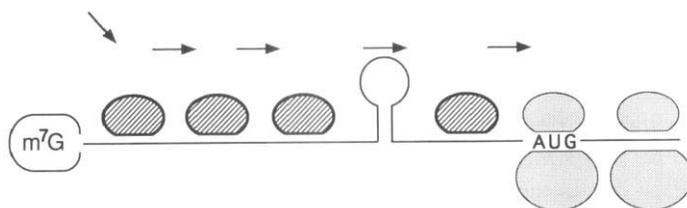


FIG. 1. A scheme for the "scanning" model of translation initiation. The 43-S preinitiation complex (hatched) binds the mRNA at the 5' end and scans linearly in a 5'→3' direction (arrows) until it reaches the AUG start codon. Association of the 40-S and 60-S ribosomal subunits is followed by the onset of polypeptide synthesis. The stem-loop represents a moderately stable secondary structure within the 5' UTR which is readily melted by the preinitiation complexes. The cap-binding complex is bound to the 7-methyl-guanosine (m⁷G) cap.

tion and subsequently associates with eIF-4A and p220, or whether it binds as a subunit of a preassembled eIF-4F complex. The next proposed step is unwinding of secondary structures in the mRNA by eIF-4A/4B in an ATP-dependent manner. The 43-S subunit binds to the "structureless" mRNA and begins to scan toward the AUG. A moderate hairpin structure in the 5' UTR can be readily passed by the scanning 43-S complex, but more stable secondary structures ($\Delta G \leq -50$ kcal/mol) block translation initiation. This impediment by the secondary structure is counterbalanced in cells overexpressing the cap-binding protein eIF-4E (31). Overexpression of this factor in NIH 3T3 cells and rat 2 fibroblasts led to malignant transformation, growth in soft agar, and tumor formation when the cells were injected into nude mice (66). A mutant eIF-4E protein that could not be phosphorylated at Ser-53 had no transforming potential. Thus, eIF-4E can be considered a novel type of cytoplasmic proto-oncogene (67). Several questions with regard to the scanning model remain open. Where precisely does the 43-S complex bind to the mRNA? Does the 43-S complex have an inherent ability for scanning? Finally, is eIF-4A/4B important after a 43-S complex has become bound to an mRNA?

Recently, translation initiation of fused transcripts bearing the 5' UTR of cauliflower mosaic virus mRNA has been analyzed in plant protoplasts. These experiments yielded results that were unexpected in view of the scanning model (68). The authors suggest that the preinitiation complex joins the mRNA in a cap-dependent manner and begins to scan toward the AUG start codon until it encounters a region of stable secondary structure containing multiple AUGs. It bypasses this region by moving in a nonlinear fashion to downstream neighboring sequences and then continues scanning (ribosome "shunt").

2. REINITIATION

As mentioned above, the ribosome initiates translation at the first AUG codon unless the context is unfavorable. When "leaky scanning" occurs, the preinitiation complex continues to search for the next favorable AUG. Another possibility exists for the ribosome to initiate translation at a downstream AUG. When a mini-cistron precedes the major ORF, both ORFs may be translated in a process called "reinitiation."

Multicistronic mRNAs are common in bacteria and downstream cistrons can be translated with high efficiency. In eukaryotic cells, no natural *bona fide* bicistronic messages have been found so far. However, mRNAs can contain one or more small ORFs upstream of the initiation codon. If the mini-cistron is not translated, the major ORF is translated by a leaky scanning mechanism. If it is translated, the major ORF has to be translated by a reinitiation mechanism. In the latter case, the translational efficiency is reduced compared to a similar message lacking an upstream mini-cistron.

The mechanism of reinitiation is not well understood. It is thought that scanning can resume after translation of a short uORF and reinitiation occur at the next AUG. Hypothetically, some initiation factors remain bound to the 40-S subunit during the translation of a mini-cistron. Thus, after termination of translation of the short uORF and dissociation of the 60-S ribosomal subunit, the remaining "semi-preinitiation complex" may recruit the missing components and be able to reinitiate thereafter (see Fig. 2 for a simplified scheme). The positioning and length of the reading frames influence the reinitiation efficiency. As one would predict, extending the distance between the mini-cistron and the major ORF increases the reinitiation efficiency (39), while long uORFs drastically reduce downstream cistron translation. A prominent example for translational regulation by a reinitiation mechanism is that of the yeast GCN4 mRNA. Its four uORFs serve as regulatory elements that

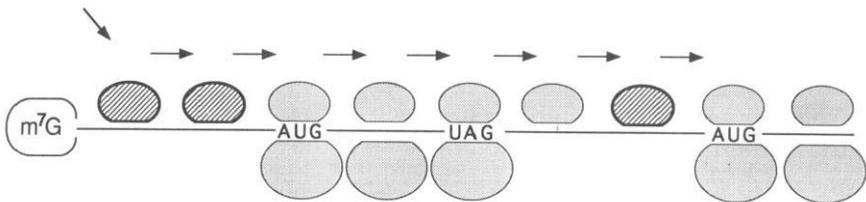


FIG. 2. A scheme for translation reinitiation. The 43-S preinitiation complex (hatched) scans the 5' UTR (arrows) and stalls at the first AUG start codon. Following translation of a short uORF, the 40-S ribosomal subunit, in a form of a "semi-preinitiation" complex, resumes scanning, and, on the way to the AUG start codon of the major ORF, becomes reinitiation-competent (hatched). Reassociation of the 40-S and 60-S ribosomal subunits is accompanied by translation of the major ORF.

modulate the translation of GCN4 in response to amino-acid starvation (for details, see Section II,A).

3. INTERNAL INITIATION

While the scanning model can account for translation initiation of the vast majority of eukaryotic messages, some viral messages do not possess a cap structure at the 5' end and must be translated by a cap-independent mechanism. These unusual mRNAs belong to the animal picornavirus family. The genomic RNAs that encode a polyprotein share a common feature: they have a very long (600- to 1200-nucleotide) 5' UTR with a high degree of secondary structure, and several AUGs (52). According to the scanning model, an uncapped structure-rich and upstream AUG-burdened mRNA would have to be translated extremely poorly. The finding that picornaviral RNAs are translated efficiently in infected cells has prompted new ideas about their mechanism of translation.

The first evidence for cap-independent internal initiation of translation came from poliovirus and encephalomyocarditis virus (EMCV). Polioviral infection causes a drastic reduction in host protein synthesis and preferential translation of the viral mRNA (for reviews, see 69, 70). The failure to translate the capped cellular mRNAs is associated with proteolytic cleavage of p220 by a virus-encoded protease (71, 72). However, the proteolysis of p220 is not sufficient to cause complete inhibition of host protein synthesis (73). A long segment of the 5' UTR of the poliovirus RNA (region P) is necessary and sufficient to confer internal initiation to (bicistronic) indicator constructs (74–76). Small deletions within this sequence result in translation-deficient mutants, indicating that preservation of a large region is crucial for function.

The sequences that allow the ribosome to bind to the mRNA downstream of the cap were initially called ribosomal landing pads (RLPs) and are now mostly known as internal ribosomal entry sites (IRESs). An experimental strategy to identify a functional IRES is to insert the putative IRES sequence into artificial bicistronic transcripts between the ORFs for two indicator proteins, such as thymidine kinase and chloramphenicol acetyltransferase (CAT), and test for their translation in transfected cells or in cell-free extracts. The upstream cistron should be translated by a cap-dependent scanning mechanism, while the downstream one should be translated by ribosomes directed to an internal entry site 5' of the downstream cistron. Translation of the downstream cistron should be unaffected by inhibiting the cap-dependent translation of the upstream cistron (poliovirus infection, insertion of stable secondary structure into the 5' UTR). Using this approach, several investigators have identified IRESs in the mRNAs of poliovirus, foot-and-mouth-disease virus (FMDV) (77, 78), EMCV (79–82), and rhinovirus (83). These viruses are all members of the animal picornavirus family. An

IRES has also been identified in the tricistronic transcript encoded by infectious bronchitis virus (84), a member of the coronavirus family.

Internal initiation seems to be a mechanism not restricted to viruses. The fact that a few cellular polypeptides, such as the glucose-regulated 78-kDa protein (GRP-78 or BiP) is synthesized in poliovirus-infected cells suggests that their messages might be translated in a cap-independent manner. The 5' UTR of GRP-78 mRNA indeed functions as an IRES in bicistronic transcripts (85). Another example of a cellular mRNA containing an IRES is the homeotic gene *Antennapedia* from *D. melanogaster* (86). Internal ribosome binding is conferred by a 252-nucleotide segment, which is the shortest functional IRES identified so far. A 55-nucleotide region within this segment, found to be conserved between different *Drosophila* species, appears to be sufficient as an IRES in cultured *Drosophila* cells (54). The biological roles of the IRESs of GRP-78 and *Antennapedia* remain to be defined, particularly as both mRNAs are monocistronic.

To avoid misinterpretations and unambiguously to demonstrate that a particular sequence functions as an IRES, it is necessary to show that the downstream cistron is translated as *part of* a bicistronic mRNA, that is, it remains intact, and it is associated with polysomes under conditions in which the upstream cistron is not translated. This is indeed the case for the poliovirus (76) and GRP-78 (86) IRESs. Further experimental data in support of the internal initiation model are the following. Transfection of uncapped bicistronic transcripts containing a functional encephalomyelitis virus IRES into cells results in translation of only the second cistron (87). Furthermore, translation of the first, but not the second, cistron of a bicistronic transcript can be competed out by a cap analog in a cell-free system (88). All these results show that internal initiation is unrelated to the translation of the first cistron and thus distinguish it from a reinitiation mechanism.

The IRESs identified so far require a minimal segment of ~450 nucleotides in order to function (with the exception of the *Antennapedia* IRES), and exhibit relatively little sequence similarity to each other (52). A common feature found in all picornaviral IRESs is a conserved polypyrimidine tract located at a 5'-proximal position to the AUG initiation codon (89). This polypyrimidine stretch was shown to be essential for internal initiation, since its deletion gives rise to nonfunctional mutants (90, 91). Disruption of the spatial arrangement by insertion or deletion of short segments between the polypyrimidine tract and the AUG codon abolishes ribosomal entry, and revertants show restoration of this arrangement (92, 93).

It is not clear in which form the initiation apparatus makes the first contact with the mRNA. Conceivably, it is the 43-S preinitiation complex. It is also not known exactly which subsequent steps are required to assemble a translation-competent 80-S ribosome on the initiation codon. In fact, experi-

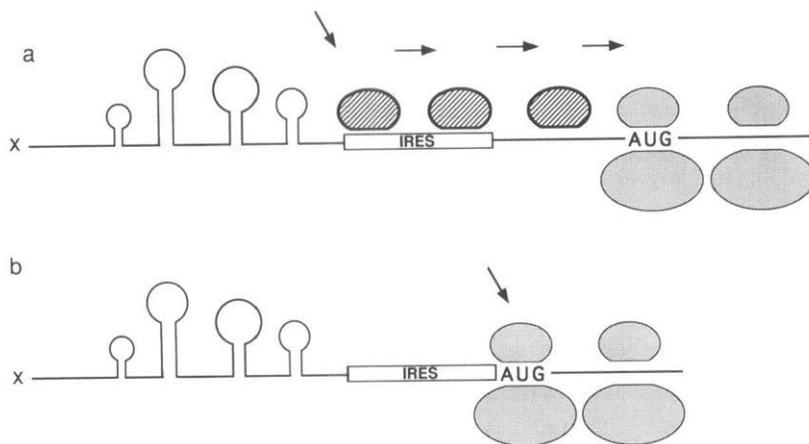


FIG. 3. Models for "internal initiation" of translation. (a) The preinitiation complex associates with the mRNA at an "internal ribosomal entry site" (IRES) and subsequently scans linearly in a 5'→3' direction (arrows) to reach the AUG start codon. (b) The preinitiation complex (or the 40-S ribosomal subunit alone) directly binds to the AUG start codon, which is located adjacent to the IRES. The stem-loops represent a highly structured 5' UTR. X, 5' end not bearing an m^7G cap.

mental evidence suggests that this may not follow the same path in all cases. Since the IRESs of polio- and rhinoviruses are positioned at considerable distance upstream of the initiator AUG, it has been suggested that the preinitiation complex scans the segment between the binding site and the AUG in a 5'→3' direction (76) (Fig. 3a). On the other hand, the IRESs of EMCV and FMDV include the initiation codon, and the ribosome seems to bind directly to the AUG (94) (Fig. 3b). In addition to these differences, translation of polio- and rhinovirus RNAs is poor in rabbit reticulocyte lysate and gives rise to aberrant products, whereas EMCV and FMDV RNAs are translated efficiently and correctly (52). The fact that efficient translation of polio- and rhinovirus mRNAs in rabbit reticulocyte lysate is restored upon addition of HeLa cell extracts indicates that different requirements for cellular factors exist between the two sets of messages.

Are cellular factors other than known initiation factors involved in internal initiation? A few proteins have been shown to form specific complexes with IRES segments (95–97), but their functional involvement in internal initiation is still unclear. Interestingly, eIF-2 was identified among the cellular proteins that bind to sequences of the poliovirus 5' UTR (98). A protein of ~52 kDa (p52) from HeLa cell extracts, previously shown to specifically crosslink by UV irradiation to the poliovirus 5' UTR (99), was recently found to stimulate poliovirus translation. Partial sequence analysis revealed that

p52 is identical to La autoantigen, and addition of recombinant La protein was shown to stimulate and restore accuracy of the translation of poliovirus RNA in reticulocyte lysate (100). Another protein (56 kDa) has been identified for its ability to bind specifically to the polypyrimidine tract of IRESs (see also Section II,C).

Clearly, all modes of translation initiation (cap-dependent scanning, reinitiation, and internal initiation) are mechanistically far from being understood and warrant further characterization.

II. Selective Translational Regulation via 5' UTR Elements

In the previous section, the basic aspects of translation initiation, as well as models for how an mRNA is bound by the ribosome, were outlined. In addition to the role of the 5' UTR in translation initiation in general, *cis*-acting sequences in the 5' UTR of specific mRNAs constitute major regulatory elements for selective translational control. Very interesting mechanisms of mRNA-specific translational regulation via 3' UTR sequences have also been described (reviewed in 101 and 102), but discussion of them is beyond the scope of this review.

Regulatory elements within the 5' UTR of mRNAs are usually identified by linking the 5' UTR to indicator constructs and demonstrating regulation of the fused mRNA. Subsequently, deletion analysis serves to identify the minimal sequence(s) *sufficient* for translational control. In a complementary approach, mutagenesis of this sequence in the context of the authentic mRNA is used to demonstrate the *necessity* of the element(s) for regulation. Once the *cis*-acting sequences are known, the search for *trans*-acting cellular factors begins, usually biochemically by RNA-binding assays or genetically.

A. GCN4

The GCN4 protein is a positive transcriptional regulator of many genes involved in the biosynthesis of amino acids and purine in the yeast *S. cerevisiae*. An elaborate control mechanism regulates GCN4 expression in response to amino-acid and purine availability at the translational level. Four short uORFs in the 5' UTR of GCN4 mRNA cause inefficient translation of GCN4 mRNA in amino-acid-replete cells, but allow translational activation when amino acids or purines are limiting (for reviews, see 103 and 104; see also 105). The regulatory elements confer the same pattern of expression when placed in the 5' UTR of heterologous yeast transcripts (106). The first

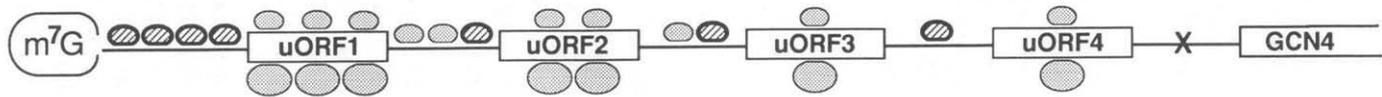
and fourth GCN4 uORFs are crucial for function, whereas the other two seem to play a minor role, since their deletion or point mutations have little effect on GCN4 regulation (107, 108).

In general, uORFs impair the translational efficiency for the major ORF, unless the distance separating the uORF and the translation start site is long (see Section I,C). In GCN4 mRNA, the presence of uORF 1 alone reduces translational efficiency by about 50%, while uORF 4 alone almost abolishes GCN4 translation. To bypass this barrier, the combination of uORF 1 with uORF 4 with the correct spacing is absolutely required, and uORF 1 serves under these conditions as a positive regulatory element in amino-acid-starved cells. The positioning of uORFs 1 and 4 with respect to each other and the GCN4 ORF is also critical for regulation, since the two uORFs placed in different order result in a repressed phenotype (109). The length of the uORFs, rather than any specific sequences or structural elements, seems to be crucial, because regulation is primarily affected by perturbing the length, and only to a lesser degree by point mutations or replacement with heterologous nucleotides within them (109–111).

During the course of translation initiation of GCN4 mRNA, uORF 1 is translated and, after termination, scanning resumes. Under repressing conditions (when amino acids or purines are plentiful), translation will reinitiate at the next three uORFs. After translation of uORFs 2, 3, and particularly 4, it progressively loses the potential to reinitiate at the GCN4 start codon (Fig. 4a). Under derepressing conditions, when amino acids are limiting, the scanning complex will “ignore” the downstream uORFs and traverse the leader sequence until it reaches the GCN4 start codon. In molecular terms, this is a result of a crucial component for reinitiation being missing, and only recruited on the way toward the GCN4 AUG, after the uORFs have been bypassed (Fig. 4b). The prediction that uORF 4 is not translated under derepressing conditions is supported by the finding that extension of its length to 130 nucleotides in such a way that it overlaps with the first codons of GCN4 has virtually no effect on GCN4 translation (112). An internal initiation mechanism appears unlikely, since insertion of stable secondary structures upstream and downstream of uORF 4 strongly diminishes GCN4 translation (112).

A central aspect in the regulation of GCN4 expression is the role of GCN2, a factor first identified genetically to stimulate GCN4 translation in amino-acid-starved cells. Cloning and sequence analyses of GCN2 revealed two domains, one with striking homology to serine and threonine kinases and the other, to His-tRNA synthetases (113). The kinase domain is closely related to the double-stranded RNA-activated inhibitor (DAI) and the heme-controlled repressor (HCR), two well-characterized mammalian kinases in-

a



b

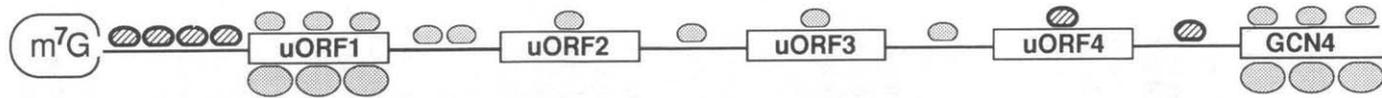


FIG. 4. (a) Repression of GCN4 mRNA translation. As a result of translation of all four uORFs, no reinitiation-competent 40-S ribosomal subunit (hatched) reaches the AUG start codon of the GCN4 ORF. (b) Derepression of GCN4 mRNA translation in amino-acid-starved cells. Following translation of uORF 1, the subsequent uORFs are bypassed and reinitiation competent 40-S ribosomal subunits reach the AUG start codon of the GCN4 ORF.

volved in global translational regulation under stress conditions. Both enzymes phosphorylate eIF-2 α , thereby blocking eIF-2/GDP recycling by eIF-2B (GEF), and thus shutting off protein synthesis (114, 115 and references therein). A conserved lysine residue in the active site of DAI and HCR is also found in the kinase domain of GCN2 (Lys-559). Substitution of Lys-559 with arginine or valine abolishes GCN2 regulatory function (116), indicating that the kinase activity is involved in the activation of GCN4 translation. GCN2 is also inactivated by mutations of the His-tRNA synthetase domain (116), suggesting that this region is critical for GCN2 function and that it regulates the kinase activity. Since the His-tRNA synthetase binds uncharged tRNA, which is abundant in amino-acid-starved cells, it has been proposed that GCN2 senses amino-acid availability through this domain such that binding of uncharged tRNAs results in kinase activation. Phosphorylation of a protein factor involved in (GCN4) translation by GCN2 would derepress GCN4 synthesis (117).

GCN2 kinase is a ribosome-associated enzyme (118), and its substrate was identified *in vivo* and *in vitro*, by genetic and biochemical means, to be eIF-2 α (119). Previous experiments had already demonstrated that GCN4 translational derepression correlated with partial eIF-2 inactivation (120, 121). In addition, some negative regulators of GCN4 mRNA translation, the products of the GCD genes, were shown to be general translation factors (120, 122, 123). Mutation of these genes constitutively derepresses GCN4 translation. GCD1 and GCD2, which are associated together with GCN3 (a positive GCN4 regulator) in a large complex, appear to be subunits of the yeast homologue of the mammalian GTP/GDP exchange factor eIF-2B (122, 123). Furthermore, the GCD6 and GCD7 gene products are additional subunits of yeast eIF-2B (124).

These findings provide new insight into the mechanism of GCN4 mRNA translation and the role of eIF-2. In cells where amino acids are plentiful, nonphosphorylated eIF-2 α is an active component of the initiation complex. After translation of uORF 1, a new preinitiation complex, including eIF-2 α , is rapidly assembled for reinitiation. Translation of uORFs 2–4 progressively diminishes the chances for another reinitiation event. Under these conditions, translation of GCN4 mRNA can only take place in GCD mutant cells, where the mutated eIF-2B fails to regenerate efficiently the eIF-2 pool and thus impairs eIF-2 function. In cells where amino acids are limiting, a fraction of eIF-2 α is phosphorylated by GCN2 and its recycling is slowed. As a result of a partial inactivation of eIF-2 α , GCN4 mRNA is translated because rapid reinitiation is less likely, and therefore, uORFs 2–4 are bypassed. Under these conditions, reinitiation can occur when the GCN4 initiation codon is reached.

B. Ferritin and Erythroid 5-Aminolevulinate Synthase (eALAS)

A very different mechanism of translational control by regulatory elements in the 5' UTR of an mRNA operates in many animals for the regulation of mRNAs involved in iron metabolism. The transcripts encoding the iron storage protein ferritin and eALAS, an enzyme catalyzing a rate-limiting step in heme biosynthesis, are controlled by interactions of a regulatory protein with the mRNAs. A conserved regulatory element called an "iron-responsive element" (IRE) is found in the 5' UTRs of ferritin (H- and L-chains) (125, 126) as well as eALAS mRNAs (127, 128). The typical features of an IRE, which is ~30 nucleotides long, include a six-membered loop with the sequence 5'-CAGUGN-3', and a base-paired region of somewhat variable length, which is interrupted by an unpaired C residue located exactly 5 nucleotides upstream of the first C of the loop. A protein that binds to IREs, the "iron regulatory protein" (IRP), is a cytoplasmic polypeptide formerly known as "iron regulatory factor" (IRF) (129), IRE-binding protein (IRE-BP) (130), ferritin repressor protein (FRP) (131), or P-90 (132). IRP is the molecular sensor of the iron levels in the cell, and has two affinity states for IREs. When cells are iron-deficient, IRP binds with high affinity to the IREs in ferritin and eALAS mRNAs, and blocks translation. When iron is plentiful, IRP has a low IRE-binding affinity, resulting in derepression of ferritin and eALAS mRNA translation. The molecular details of how IRP senses the cellular iron levels and "translates" it into different RNA-binding characteristics are beginning to emerge and have recently been reviewed (133, 134).

The control of gene expression in response to a physiological stimulus by the reversible interaction of a repressor protein with its cognate mRNA sequence located at the 5' UTR of a message provides a fairly simple mechanism for gene-specific translational regulation, reminiscent of the classic repressor/operator systems in prokaryotes. The case of ferritin was the first such system described in eukaryotes (135). Ferritin translational regulation is not cell-type-specific, and the IRE is phylogenetically conserved from frog to human. The IRE-IRP interaction is necessary and sufficient to control ferritin and eALAS mRNA translation, both *in vivo* and in cell-free systems (136-139). Deletion of the regulatory element from the 5' UTR or point mutations that prevent IRP binding cause constitutively derepressed translation, indicating that IRE acts in the 5' UTR of an mRNA as a negative regulatory element.

The mechanism by which the IRE-IRP interaction inhibits translation is only partially understood. The position of the IRE with regard to the cap structure is conserved in all ferritin and eALAS mRNAs. When the distance between the IRE and the cap is increased by introducing "spacer" RNA

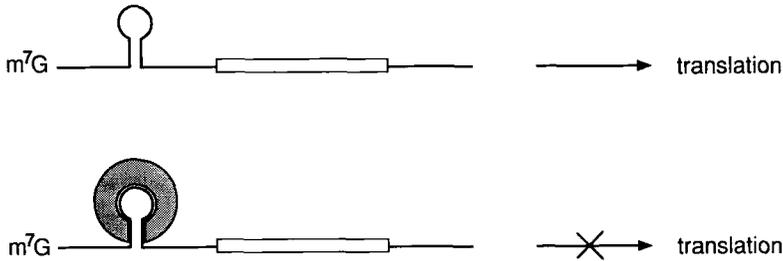


FIG. 5. Repression of translation by RNA-protein complexes within the 5' UTR of the mRNA.

sequences, iron regulation and the translational inhibition by the IRE-IRP interaction are gradually reduced (140). An IRE within ~ 40 nucleotides from the cap, with respect to the unpaired C, can bind IRP and inhibit translation. An IRE located further downstream in the 5' UTR retains the ability to interact with IRP, but the IRE-IRP complex in this position is not sufficient to exert an inhibitory effect. It remains to be defined whether the initiation machinery proceeds by dissociating or bypassing the RNA-protein complex.

The IRE-IRP complex appears to block translation initiation sterically, because the translational repression can be mimicked by other RNA-protein interactions in the 5' UTR of indicator mRNAs. RNA-binding proteins with physiological functions unrelated to eukaryotic translation, such as the bacteriophage MS2 coat protein or the snRNP U1A protein, have been evaluated as translational repressor proteins by placing their cognate RNA-binding sites near the cap of indicator transcripts (141). It could be demonstrated that the RNA-protein complex leads to translational arrest *in vitro* (Fig. 5). Similar results have subsequently been obtained in *S. cerevisiae* and transfected HeLa cells (R. Stripecke and M. W. Hentze, unpublished data). These results support a general steric hindrance model for translational inhibition by RNA-protein interactions within the 5' UTR. Further work must define which step in the translation initiation pathway is blocked by IRE-IRP and other RNA-protein complexes.

C. Other Systems

1. RIBOSOMAL PROTEINS

Ribosome assembly requires coordinated biosynthesis of four rRNA species and 70-80 ribosomal proteins (r-proteins). The expression of rRNA and r-proteins correlates with the requirement for protein synthesis at different rates of growth, and is modulated by a variety of control mechanisms. Regulation of the synthesis of r-proteins in eukaryotes differs from that in *Es-*

Escherichia coli, in which translation can be controlled by binding of various r-proteins to their own mRNAs in an autoregulatory feedback mechanism (for a review, see 142). The eukaryotic ribosome biosynthesis pathway is particularly interesting in germ-line cells of many animal species, where dramatic changes in cellular translation occur at different developmental stages. In *X. laevis*, ribosome biosynthetic activity is very high during oogenesis, declines during meiotic maturation, and resumes during early embryogenesis (for a review, see 143). A considerable number of maternal mRNAs, including those encoding r-proteins, can be stored as translationally inactive RNPs in the cytoplasm of *Xenopus* and several marine invertebrate oocytes (144).

The developing *X. laevis* oocyte and embryo provide an experimental model system to study the regulatory mechanisms governing ribosome biosynthesis. In this system, the expression of r-proteins is modulated post-transcriptionally at the levels of mRNA processing and mRNA translation (145). In addition to *Xenopus*, r-protein synthesis appears to be regulated translationally under a variety of conditions in different cell types and species, such as in growth-stimulated versus -arrested mammalian cells (146, 147), in developing *Dictyostelium discoideum* (148), or in *Drosophila melanogaster* (149). In all cases examined, r-protein mRNAs are deposited in the cytoplasm as translationally inactive mRNPs and are only recruited for translation in response to an increased cellular need for ribosome biosynthesis.

The regulatory *cis*-acting elements are located in the 5' UTR of the mRNAs. Deletion of the 5' UTR of murine r-protein L32 mRNA, or substitution of the 5' UTR with that of actin, renders the mRNA constitutively active in translation (150). When the 5' UTR of *Xenopus* r-protein S19 mRNA was fused to a CAT reporter gene and microinjected into oocytes, it conferred developmental regulation to CAT translation resembling that of the endogenous S19 mRNA (151). The same conclusion was reached from experiments in which sequences of the 5' UTRs of murine r-proteins L30 and S16 mRNAs were fused to a human growth hormone (hGH) indicator and transfected into P1798 mouse lymphosarcoma cells (150). Translation of S16 mRNA is also regulated during mouse myoblast differentiation, and, consistent with the results described above, regulation of hGH expression depends on the presence of the first 31 nucleotides of the 5' UTR of S16 mRNA (152).

The r-protein L32 of *S. cerevisiae* regulates translation of its own mRNA *in vivo*, and the 5' UTR of the mRNA is involved in this regulation (153). Expression of an L32 mRNA in which the 5' UTR was placed with a heterologous sequence resulted in L32 r-protein accumulation. Furthermore, co-expression of a *LacZ* transcript fused to the 5' UTR of L32 mRNA together

with high amounts of L32 r-protein markedly inhibited β -galactosidase synthesis. Additional *in vitro* studies are required to demonstrate a direct interaction of L32 r-protein with a putative binding site in the 5' UTR of its mRNA, and to unravel this interesting regulatory mechanism.

Common features of the mRNAs encoding r-proteins include relatively short 5' UTRs (35–50 nucleotides) and an oligopyrimidine tract of 8–14 nucleotides near the cap site (89, 154, 155). Deletion of this tract or replacement of pyrimidines with purines derepresses translation (150, 156), suggesting a functional importance for the conserved oligopyrimidine sequences. Moreover, displacement of the oligopyrimidine tract of S16 mRNA to a site further downstream of the cap also abolishes translational regulation, indicating a positional requirement. A 56-kDa protein specifically crosslinks *in vitro* to the 5' UTR of murine L32 mRNA; it fails to interact with a mutated 5' UTR lacking the oligopyrimidine tract (156).

Four polypeptides were shown to bind to regions of the 5' UTR of *Xenopus* L1 mRNA (157). Two of them bind to the oligopyrimidine tract and, interestingly, one of them has a molecular mass of 57 kDa. It is not known whether the involvement of a polypyrimidine tract in internal initiation of translation of picornavirus mRNAs and the role of a polypyrimidine motif in the regulation of r-protein mRNA translation are functionally related. Conceivably, some *trans*-acting factors might be shared. However, the position-dependence of the polypyrimidine tract in r-protein mRNAs argues against internal initiation as the mechanism by which these mRNAs are translated. Clearly, more experimental data are needed to define the role of *trans*-acting factors in the translational regulation of r-proteins.

2. PROTEINS RELATED TO GROWTH CONTROL

The mRNAs encoding some of the vertebrate proteins involved in growth control and signal transduction (proto-oncogene products, transcription factors, growth factors, and their receptors) contain unusual 5' UTRs. These UTRs are characterized by many features that normally render a message poorly translatable: they are remarkably long, are highly structured, and often carry several upstream AUGs or short uORFs (for a review, see 22). Although some of the reported noncoding sequences might represent cDNA cloning artifacts derived from partially unprocessed mRNA precursors, the fact remains that mRNAs for growth-related proteins are frequently furnished with these unusual leaders. To elucidate the functional significance of these features for specific regulation, the 5' UTRs of several proto-oncogene mRNAs have been introduced into heterologous genes and examined for their effect on translational efficiency *in vitro* as well as in cell culture.

The proto-oncogene *c-myc* is a DNA-binding protein with fundamental importance for the control of cell growth. The 5' UTR of murine *c-myc*

mRNA inhibits translation of indicator constructs *in vitro* (158) and *in vivo* after microinjection into *Xenopus* oocytes (159). The region responsible was identified as residing within a 240-nucleotide segment. However, *in vitro* translation of the same constructs in HeLa cell extracts as well as transfection studies into several established cell lines such as COS-1, S194, 70Z/3, PD31, 38B9 (mouse pre-B cells), U937 (human monocytic cells), NIH 3T3, or P19 (mouse teratocarcinoma) failed to reproduce this inhibitory effect of the *c-myc* 5' UTR (159). It appears that the function of the 240-nucleotide segment is host-dependent. Expression of *c-myc* in *Xenopus* oocytes is reduced during oocyte maturation, and the protein accumulates after fertilization during early embryogenesis (160, 161). The 5' noncoding sequences of *c-myc* mRNA seem to be involved in this developmental control mechanism, because microinjection of *in vitro*-transcribed reporter mRNAs carrying the *c-myc* 5' UTR into oocytes and early embryos reproduced the differential expression pattern without affecting mRNA stability (162).

Another interesting aspect of the translation of *c-myc* mRNA is the induction of translation initiation at an upstream non-AUG codon in response to methionine deprivation. This effect was observed in confluent cultures of murine and avian cells that synthesized an N-terminally extended *c-myc* polypeptide (163). Similar cases of non-AUG initiation, especially of genes related to growth control, have been reported (164–166), but *c-myc* is the first documented example of utilization of an alternative initiation codon in response to nutrient starvation in higher eukaryotes. One wonders whether a modification of initiation factors involved in AUG recognition, possibly eIF-2 (see Section I,B), might be related to the regulated switch of the *c-myc* start codons.

Platelet-derived growth factor (PDGF) is a basic protein composed of two chains, α and β . The β polypeptide is identical to the product of the *c-sis* proto-oncogene, the cellular homologue of the retroviral *v-sis* oncogene. The mRNA for *c-sis*/PDGF β contains a long 5' UTR that is about 70% (G+C)-rich and severely impairs *in vitro* translation in wheat germ extract (167). The 5' UTR also impairs expression of indicator genes after transfection into cultured cells. Removal of (G+C)-enriched segments partially relieves the inhibition, and deletion of the entire 5' UTR results in a 40-fold enhancement of expression *in vivo* (168, 169). The improved translational ability of *c-sis*/PDGF β mRNA with a truncated 5' UTR correlates with an increased transforming activity (168), suggesting that the inhibitory effect exerted by the high (G+C) content is physiologically relevant.

Similar experimental approaches have been used to study the mRNAs of several other proto-oncogenes, including *c-erb A* (170), *lck* (171), and the transforming growth factor β 3 (TGF- β 3) (172). As was found for *c-myc*, the long (G+C)-rich 5' UTRs, which are burdened with upstream AUGs and

uORFs, acted as potent translational inhibitors. However, there is currently no evidence for an involvement of these uORFs in translational regulation.

Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the biosynthesis of polyamines, cationic molecules essential for the growth of prokaryotic and eukaryotic cells. Expression of ODC is induced by various mitogens and growth factors, and several mechanisms cooperate in the regulation of ODC activity. Characterization of ODC cDNA and genomic clones revealed sequence homologies among different species that extend from the protein-coding region into the noncoding region. The 5' UTRs are approximately 300 nucleotides long, are (G+C)-rich, and have the potential to form extremely stable stem-loop structures. The 5' noncoding regions of rat and hamster ODC mRNAs have been inserted into heterologous genes and were found to impair translation *in vitro* and *in vivo* (173, 174). Since the same effect was noticed when the hamster 5' UTR sequences were placed in the inverse orientation, the repression appears to be a consequence of the secondary structure rather than the primary nucleotide sequence. Interestingly, the ability of ODC mRNA to be translated in renal tissue was found to differ markedly between the two murine species *Mus domesticus* and *Mus pahari*. This difference correlated with small differences in the nucleotide sequences of the 5' UTRs of the ODC mRNAs, which destabilize the predicted secondary structure (175).

Are the mRNAs coding for onco-proteins and growth factors translated by the typical scanning mechanism? As the scanning model would predict, the mRNAs carrying these complex 5' UTRs are translated very poorly. This inefficiency probably reflects the physiological need to keep the expression of these proteins restricted. As discussed in Section I,A,2, earlier translation of mRNAs with highly structured 5' UTRs is improved in cells overexpressed eIF-4E (31), which causes malignant transformation. It would seem plausible that eIF-4E transforms cells by facilitating the translation of oncogene mRNAs that are normally poorly translated, but this concept has not yet been investigated directly.

3. OTHER INTERESTING EXAMPLES

Thymidylate synthase is a key enzyme required for DNA synthesis. In addition to cell-cycle-dependent regulatory mechanisms known to affect the activity of the enzyme, it appears that the translation of human thymidylate synthase mRNA is negatively autoregulated by specific binding of recombinant thymidylate synthase protein to its own mRNA *in vitro* (176). The RNA-protein complex causes translational inhibition reminiscent of the example of ferritin regulation discussed in Section II,B. Translational inhibition by the protein is relieved by addition of the substrates dUMP and 5,10-methylenetetrahydrofolate, indicating that the mRNA competes with the

enzyme substrates for binding to overlapping regions of the protein (176). Two binding sites for the protein have been mapped in the thymidylate synthase mRNA. One of them resides in the 5' UTR; the other, in the coding region (177). A region of 36 nucleotides from the 5' UTR that includes the translation initiation codon and has the potential to form a stem-loop structure is protected from RNase digestion after UV crosslinking with the protein.

A similar approach has been used to investigate human dihydrofolate reductase, another enzyme with an essential role in dTMP biosynthesis. As with thymidylate synthase, this enzyme can bind specifically to its own mRNA and thereby repress its translation *in vitro* (178). The interaction of dihydrofolate reductase with its mRNA is impaired in the presence of its substrates dihydrofolate and NADPH, indicating that mRNA binding might be mediated by active-site regions. While these *in vitro* results represent an exciting extension of the theme of how mRNA-protein interactions regulate translation, their physiological significance in living cells must be demonstrated directly. From the above examples, it seems very likely that autoregulatory translational mechanisms may not be restricted to bacteria.

Expression of a small family of genes involved in spermatogenesis in *D. melanogaster* is translationally regulated by elements located in the 5' UTRs of their mRNAs. One of them, the mRNA of the *Mst87F* gene, contains a 12-nucleotide element [called a "translational control element" (TCE)] in its 5' UTR that confers translational control to heterologous transcripts *in vivo* (179). The TCE operates as a negative regulatory element and is conserved in a cap-proximal position within the mRNAs of at least six other members of the family. Complete deletion, point mutations, or placement of the regulatory sequences further downstream of the cap result in derepression of translation, which is accompanied by lengthening of the poly(A) tail. It is conceivable that the TCE constitutes a binding site for a repressor protein acting *in trans*, in a mode reminiscent of the translational inhibition mediated by IRE-IRF or other RNA-protein interactions in the 5' UTR of mRNAs (141). However, it is incompletely understood how 5' UTR sequences can affect polyadenylation in the 3' UTR of an mRNA. Identification of a putative *trans*-acting protein(s) would provide valuable information concerning this interesting question.

A very different mechanism allows the translation of the liver transcriptional activator LAP and repressor LIP from one mRNA, which contains three in-frame initiation codons. The N-terminal region of LAP harbors a transcriptional activation domain, whereas the C-termini of LAP and LIP bind to specific promoter sites (180). The context around the first AUG in the mRNA is not optimal for initiation. LAP translation is initiated mainly at the second AUG and thus contains the transcriptional activation and DNA-

binding domains. Since the LAP initiation codon itself is also in a suboptimal context, leaky scanning causes initiation also at the third AUG, which is in an optimal context (180). The resulting protein, LIP, contains only the DNA-binding domain and acts as a competitive inhibitor of LAP. Thus, two antagonistic eukaryotic regulatory proteins that differ only in their N-termini are translated from the same mRNA by a leaky scanning mechanism.

Apart from the examples described above, more evidence suggests the involvement of the 5' UTRs of a variety of mRNAs in translational regulation. The 330-nucleotide 5' UTR of S-adenosylmethionine decarboxylase mRNA contains an upstream ORF in a cap-proximal position that encodes the hexapeptide "MAGDIS." Mutational analysis of this upstream ORF revealed that preservation of the hexapeptide sequence is essential for the cell-type-specific translational regulation in lymphocytes, as it prevents the association of the mRNA with polysomes (181). The mRNA of the maize transcriptional activator Opaque-2 contains three upstream ORFs that are involved in translational regulation by reducing translational efficiency in a fashion perhaps akin to the function of the uORFs in GCN4 mRNA (182).

The previous sections are intended to support the idea that various distinct mechanisms for translational control operate through elements contained in the 5' UTRs of mRNAs. However, only in the exceptional case of GCN4 mRNA do we currently have a reasonably detailed understanding of the molecular details of the underlying phenomena.

III. Antisense Technologies

In the 1970s, when the sequences of cellular and viral mRNAs were beginning to be elucidated, long antisense cDNAs and short chemically synthesized antisense oligonucleotides were applied to specifically prevent mRNA translation (183–185). The discovery of regulation of gene expression by natural antisense RNAs (186–189) further spurred the application of antisense techniques to turn off translation *in vitro* and *in vivo*.

After the potential benefits of antisense strategies to inhibit gene expression had become apparent, the main aim of antisense research focused on the development of "antisense" drugs for the treatment of diseases of genetic and viral origin (191, 195). As a result, the methods to synthesize natural and modified oligonucleotides chemically progressed substantially (192–194). The groundwork of selecting the most useful types of oligonucleotides with respect to their application, either as (therapeutic) inhibitors of gene expression (191, 193) or as a research tool in molecular biology, is an ongoing process (195).

In principle, it is possible to study the process of mRNA translation by

using an antisense approach. For this to be successful, two main criteria must be met. First, the antisense oligonucleotide must interfere with some step of mRNA translation, and second, the translationally arrested mRNA must remain intact. In addition, the oligonucleotides must possess suitable properties with regard to water solubility and efficient target RNA binding, and furthermore, give minimal *non*-specific effects on translation. They also must be chemically stable, resistant against nucleases, and, for studies in cell culture, efficiently internalized. In this section we describe different types of oligonucleotides fulfilling at least some of these criteria, occasionally including new types of oligonucleotides that have not yet been fully characterized, but that appear promising in this respect. Several excellent reviews have recently been published on the general use of oligonucleotides as molecular tools to turn off or to regulate gene expression (196–199) as well as on the use of antisense RNA for the same purpose (184, 200–203). We therefore focus our discussion on the applications of antisense technologies to the study of mRNA translation. However, as an introduction, we summarize relevant information concerning the chemical, biochemical, and biophysical properties of various oligonucleotides.

A. Types of Oligonucleotides

The production of oligonucleotides (oligos) containing combinations of over 100 differently modified nucleotide building blocks (192) has been made possible by recent developments in nucleotide chemistry and solid-phase synthesis. The chemical details have recently been extensively reviewed (192, 193, 204–207).

The possibility of modifying the nucleotide building block at a variety of positions is evident from Fig. 6. In order not to disturb the (information-carrying) hydrogen-bonding capability, efforts have mainly been focused on the ribose-phosphodiester backbone, which determines most of the biochemical and biophysical properties of an oligo. Heterologous chemical groups have also been linked to oligos to improve certain properties or to add new ones (208).

1. PHOSPHATE-MODIFIED OLIGONUCLEOTIDES

The first modifications of the backbone phosphate were introduced starting from normal phosphodiester DNA (PO-DNA) oligonucleotides. Phosphorothioate-DNA (PS-DNA) (198, 209–214) and phosphorodithioate-DNA (PS₂-DNA) (193, 215, 216) oligos are made by substitution of one or both of the nonbridging phosphate oxygens with sulfur (Fig. 6a). PS-DNA and PS₂-DNA oligos retain most of the properties of PO-DNA, but are resistant to most nucleases. When hybridized to the target RNA, both retain the ability to activate cleavage of the RNA by RNase H. Their success as

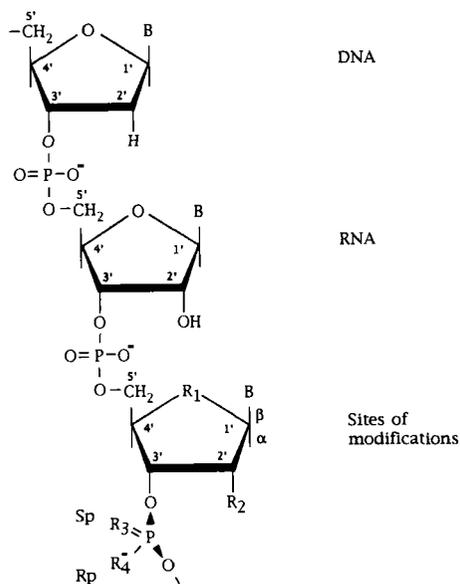


FIG. 6. Backbone modifications. The structure of natural RNA and DNA compared to phosphate-modified and ribose-modified antisense oligonucleotides described in the text.

Modifications of the phosphate			
Type	R ₃		R ₄
Phosphodiester (PO) (DNA/RNA)	O		O ⁻
Phosphorothioate (PS)	O/S		O ⁻ /S ⁻
Phosphorodithioate (PS ₂)	S		S ⁻
Methylphosphonate (MeP)	O/CH ₃		O/CH ₃
Phosphoramidate (PA)	O/NHR ^a		O/NHR ^a
Phosphotriester (PEst)	O/OR ^a		O/OR ^a
Modifications of the ribose			
Type	R ₁	R ₂	1'C
RNA	O	OH	β
DNA	O	H	β
2'-O-Me	O	O—CH ₃	β
2'-O-allyl	O	O—CH ₂ —CH—CH ₂	β
2'-F	O	F	β
Carbocyclic (4'—C)	C	H	β
4'-Thio	S	H	β
α-DNA	O	H	α

^a R, Alkyl group, etc.

specific inhibitors of gene expression is therefore not surprising, and an antisense PS-DNA oligo was the first to go on to phase II of clinical trials (191).

Charge-neutralizing modifications of the phosphate (Fig. 6a), such as the methylphosphonate (MeP-DNA) (217–221), the phosphoramidate (PA-DNA) (222, 223), and alkyl phosphotriesters (PEst-DNA) (221), also confer nuclease resistance. MeP-DNA oligos have received much attention; they are efficiently taken up into cultured cells, and inhibit translation without activation of RNase H. Oligos containing either PA-DNA or PEst-DNA have not performed as well as others in biological assays. Their chemical instability, low solubility, and considerable nonspecific effects severely limit their use as antisense reagents (221).

Oligos with other modifications are available, but little is known about their effects on translation (190–193, 224, 225).

2. RIBOSE-MODIFIED OLIGONUCLEOTIDES

The quest for nuclease-resistant oligos that hybridize to their target RNA with high affinity without affecting its stability has prompted several groups to turn to the backbone ribose. An advantage of modifying the ribose is that the charged phosphate, which determines many of the physicochemical properties, is left intact.

The 2'-O-methyl modification of RNA occurs naturally in tRNAs, snRNAs, and rRNAs and as part of the extended cap structure of some mRNAs (226, 227). This modification was the starting point for the addition of other chemical groups, for example, alkoxy groups and fluoride to the 2' position of the ribose and incorporation into oligos (Fig. 6b) (193, 228–238). These modifications enhance stability against nucleases, allow strong binding to the target RNA, and confer resistance to RNase H. Finally, they also decrease nonspecific binding of proteins to the oligo. As a result, oligos containing an allyloxy modification at the 2' position have been employed successfully in molecular biology (236, 237).

The replacement of the 4' oxygen with carbon (192, 239) (carbocyclic) or sulfur (240–242) has recently been achieved (Fig. 6b). Initial studies using oligos with these replacements have yielded promising results with respect to nuclease resistance, but, as for most new oligos, more biochemical work is required before their potential can be conclusively assessed.

The biggest change in structure involves the inversion of the base at the 1' carbon (243) (Fig. 6b). The resulting α -anomers of DNA are extremely resistant to nucleases. Oligos with this modification bind to RNA in a "syn-sense" orientation, with an affinity somewhat lower than that of normal DNA, and the formed hybrids are not a substrate for RNase H, leaving the target RNA intact.

The large number of modifications available can be combined to take full advantage of the repertoire of desirable properties. Combinatorial oligos, such as the doubly modified phosphorothioate-2'-O-Me RNA, are currently under investigation (193, 244, 245).

B. Physicochemical Properties

1. SOLUBILITY

The physicochemical properties of oligos depend on their interaction with ions, small molecules, other nucleic acids, and proteins in aqueous solutions. Natural DNA and RNA are readily soluble in water, mainly due to their negatively charged backbone phosphates. Modifications involving removal of the charge, such as the MeP-DNA, PA-DNA, and PEst-DNA (Fig. 6a), therefore yield oligos with drastically reduced water solubility. Charge-neutral oligos can, on the other hand, more easily traverse hydrophobic biological membranes, an attractive feature discussed below (Section III, C). The solubility of otherwise hydrophobic oligos (e.g., MeP-DNA) can be improved by adding hydrophilic nucleotide building blocks, such as PO-DNA or PS-DNA (217). The solubility of one type of PA-DNA oligos serially replaced by normal phosphates has also been investigated (246). The results corroborate the importance of the charged phosphate as the main determinant of water solubility.

Oligos with charged phosphates intact, but containing hydrophobic groups internally or at the ends, have altered solubility in organic solvents, affecting their experimental handling. Oligos containing 2'-O-allyl groups (Fig. 6b) are, for instance, soluble in ethanol (but not in butanol). Addition of hydrophobic tails purposely to increase oligo lipid solubility and cellular membrane permeability is discussed below (Section III, C).

2. TARGET RNA BINDING

The oligo must hybridize to its target sequence and remain bound for some time to exert its function. The two main factors that influence the binding efficiency are the (calculated) T_m of the hybrid and the presence of *intra*-molecular secondary structures, both in the target RNA and in the oligo. Other aspects of target RNA binding that are particularly important for experiments using cellular extracts or living cells are the masking of the target RNA and/or the oligo by proteins, and the possible unwinding of the hybrid by helicases (Section III, D).

The T_m depends on the number of hydrogen bonds formed between the oligo and the target RNA as well as on the conformation of the hybrid formed, which in turn affects the stacking of the bases. By varying the length of the oligo (and the number of hydrogen bonds), the T_m can easily be

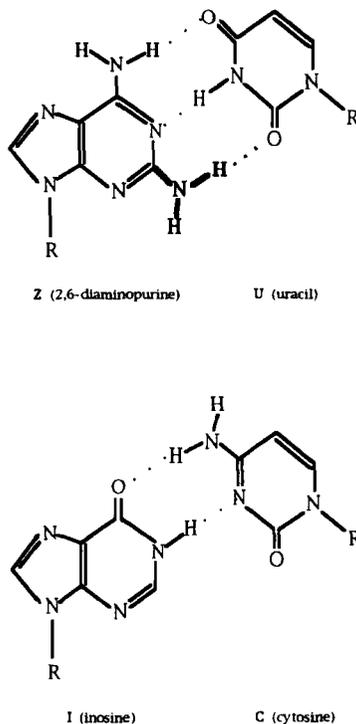


FIG. 7. Base modifications; T_m variants. Addition of one amino group to adenine (2,6-diaminopurine; Z) allows three hydrogen bonds to uracil, whereas deletion of the amino group in guanosine (inosine; I) results in base-pairing with cytosine with only two hydrogen bonds.

modulated. For use in complex mixtures of RNA (and DNA), such as in cells or cell extracts, the length of the antisense oligo is limited by possible undesirable binding to partially complementary target sites. A given sequence of 16 nucleotides is statistically not expected to occur "randomly" in the human genome ($4^{16} > 3 \times 10^9$) (247). Chemical synthesis methods are normally not limiting, because oligos between 10 and 20 nucleotides in length are sufficient for most applications.

An additional way to modulate the number of possible hydrogen bonds is to substitute inosine for guanosine and thus decrease the number of hydrogen bonds with cytosine from three to two, or to replace adenine with 2,6-diaminopurine (amino-A) (Fig. 7), which allows an extra hydrogen bond to be formed with uracil (248). The latter substitution proved to be essential for the depletion of U5 snRNA from nuclear extracts (248a). Propynyl-C- and -U-containing 2'-O-allyl oligos also have increased T_m s compared to non-

substituted oligos (250, 251). The effects of other base modifications have recently been reviewed (252).

The binding of oligos with low affinity for RNA can be increased by incorporating intercalating groups, such as acridine (253–255) and anthraquinone (256) (Fig. 8). The intercalating groups increase the T_m (but not the target specificity) of the oligo sufficiently to meet the criteria for efficient target RNA binding. The use of psoralen (Fig. 8) as an intercalating agent allows the oligo to be covalently and reversibly crosslinked to the target RNA (193, 208, 219). The higher T_m of hybrids between RNA and MeP-DNA or α -DNA oligos with such intercalating groups is a prerequisite for specific inhibition of translation by these oligos (220, 254, 257) (Section IV).

Oligos binding to RNA must compete with structures formed by the RNA itself. The most stable naturally occurring double-stranded nucleic acid is the A-form of dsRNA (12-mer, $T_m \approx 55^\circ\text{C}$) (258–260). Other very stable intramolecular RNA structures, such as tetraloops (261) and pseudoknots (260, 262, 263), play a role in translation in prokaryotes and eukaryotic viruses (264–268). Since extra energy is required to break up such stable RNA structures (269), the ideal binding site for oligos is a single-stranded region of the target RNA.

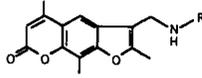
Oligo-RNA hybrids generally acquire a conformation similar to that of dsRNA (197, 213, 270, 271). DNA and DNA-like oligos all form hybrids with RNA, with a T_m lower than that of dsRNA (272). Consequently, a large excess of oligo is needed to achieve efficient occupation of the binding site. Perhaps the most unusual conformation is displayed by hybrids between RNA and α -DNA anomers (243) (Fig. 6b). The α -oligos bind in a 5'→3' (synsense) direction to the RNA. This type of hybrid has a T_m lower than that of a regular DNA-RNA hybrid, which is consistent with the poor binding of PEst-DNA to RNA, which has also been suggested to bind in a synsense direction (221).

The most stable hybrids with RNA have been achieved with oligos bearing 2'-O-alkyl groups (231, 233, 234, 237, 245, 248). A 12-mer 2'-O-methyl RNA-RNA hybrid has a T_m of 61.8°C, 5°C higher than an RNA-RNA duplex of the corresponding sequence (237). The hierarchy of T_m s for hybrids with RNA can be summarized as follows: 2'-F > 2'-O-Me > 2'-O-Et > 2'-O-allyl > 2'-O-Pro \approx RNA \gg DNA > PS-DNA \approx α -DNA > MeP-DNA (for further details, see 213, 220, 221, 236 and 243; for T_m s for hybrids with DNA, see 273 and references therein).

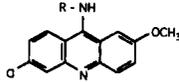
Knowledge of the exact primary sequence of the target RNA aids in the rational design of antisense oligos. *In vitro*, it is usually possible to use a large molar excess of oligo to compensate for a low T_m , provided that the employed oligo will cause few nonspecific effects on translation. If the secondary or even the tertiary structure of the target mRNA is known (274), the

Intercalation
and
x-linking
groups

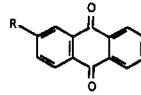
4' aminomethyl 4,5,8-trimethyl Psoralen



Acridine



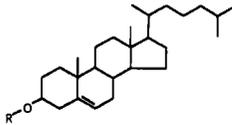
Antraquinone



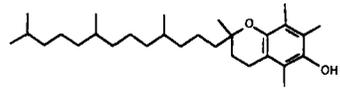
Dodecanol



Cholesterol

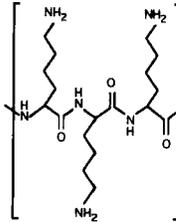


α-tocopherol (vitamin E)

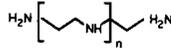


Lipophilic
groups
for
enhanced
cellular
uptake

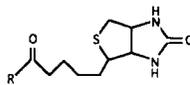
Poly L-lysine (PLL)



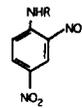
Polyamines



Biotin

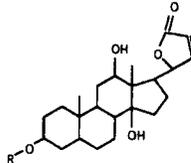


2, 4 - DNP



Affinity
groups

Digoxigenin



chances of finding an accessible binding site for the oligo are highly improved. As aids to selecting the most efficient oligo (and target site), theoretical and experimental methods have recently been developed (272, 273, 275, 276).

Regions of the target RNA containing stable stem-loop structures, formerly believed to be inaccessible to oligos, have been targeted successfully, resulting in the formation of "pseudo-half-knots" between the oligo and the RNA (269, 277) (Section IV). The formation of "guanine quartets," found in telomeres (276, 278) and triple helices, has also been reported (243, 267 and E. Pascolo, personal communication). It is therefore possible to use oligos to create hybrids that mimic many different RNA structures. A final aspect to consider in selecting an antisense oligo should be its lack of self-complementarity. Even minor self-complementarity can result in sequestration of oligos away from the target RNA (236).

3. CHEMICAL AND ENZYMATIC STABILITY

The efficacy of an oligo depends on its ability to reach its target intact. Determinants of the half-life of an oligo include its chemical stability as well as its biological stability, which is limited by nucleases and other catabolic enzymes.

Most types of oligos are stable in commonly used buffers, but "self-destruction" of oligos containing sulfur, selenium, PESt backbones, and 2'-F modifications has been reported (192, 221, 256, 279). The effect of redox state and pH in different cellular compartments on oligos containing oxidation-sensitive sulfur and selenium modifications is an aspect that has received surprisingly little attention in the literature. The problem of heavy-metal binding and subsequent hydrolysis of such oligos also must be considered.

The many different nucleases present in cells and serum could pose a major problem to the use of oligos for studying mRNA translation *in vivo*. However, the potentially destructive effects of nucleases must be seen in light of their specificities and compartmentalization. A 3'→5'-exonuclease is the most abundant nuclease activity in fetal calf serum, an important component of many cell-culture media (193, 280). Intracellularly and in cell extracts, oligos also must withstand 5'→3'-exonucleases as well as endonucleases. Tests of nuclease sensitivities of oligos normally include snake venom phosphodiesterase as an example of a 3'→5'-exonuclease, calf spleen phosphodiesterase as a 5'→3'-exonuclease, nuclease S1 as a single-stranded

FIG. 8. Pendant groups. The most commonly used pendant groups are depicted. Their addition internally or at the ends of oligonucleotides provides an efficient way of adding new functions or enhancing desired properties.

TABLE I
NUCLEASE RESISTANCE AND RNase-H ACTIVATION

Type	DNase ^a	RNase	3' exo	5' exo	Others	FCS ^b	RNase H	References
RNA	+	-	-	-		-	-	
DNA	-	+	-	-		-	+	
MeP-DNA	+	+	+	+	+(S1, mn)	+	-	217-221, 289
PS/PS ₂ -DNA	+	+	+(10 ³)	+(10 ²)	+/- ^c	+	+	211, 215, 216, 361
PA-DNA	+?	+	+(FCS)	?	?	+	-	192
PEst-DNA	+	?	+(FCS)	+(FCS)	?	+	-	221
2'-O-Me-RNA	+	+	-	+(mb)	+(S1, P1)	?	-	223, 225, 231, 236
			+(exo III)		+(mn 10 ²)			
2'-O-allyl-RNA	+	+	-	+(mb)	+(S1, P1)	?	-	225, 234
			+(exo III)					
2'-F-DNA	?	?	?	?	?	+	-	192, 225, 362
4'-C-DNA	+(S1)	?	-	(+)(10)	+(S1)	?	?	225, 239
4'-S-DNA	(-)(8)	+	(+)(76)	+	?	?	?	225, 240, 241
α-DNA	+(10 ²)	+	+(10 ²)	+	+(S1)	+	-	192, 225, 243, 363

^a The enzymes used are: DNase (deoxyribonuclease), DNase I; RNase (ribonuclease), RNase A, T1, T2, U2, or CL2; 3' exo (3' exonuclease), snake venom phosphodiesterase (SVP) or exonuclease III (exo III); 5' exo (5' exonuclease), calf spleen phosphodiesterase (CSP); others, nuclease S1 (S1), nuclease P1 (P1), mung bean nuclease (mb), and micrococcal nuclease (mn); RNase H, ribonuclease H. Numbers in parentheses refer to enhanced resistance as compared either to RNA or DNA; for example, it takes 10² longer time to digest α-DNA with DNase I than it takes to digest normal DNA.

The list makes no claims of being complete, there may be enzymes for which certain modifications are susceptible, but which have not been reported, or vice versa. ?, Not determined or not reported.

^b FCS, Fetal calf serum used alone or in tissue culture media.

^c Depending on which stereoisomer is tested, PS oligos are either fully resistant or fully susceptible to P1 or S1 (216).

endodeoxyribonuclease (and to a lesser degree 5'→3'-exonuclease and RNase), micrococcal nuclease as a sugar-nonspecific endonuclease, DNase I as an endodeoxyribonuclease, and, finally, one or more of the endoribonucleases A, T1, T2, CL3, and U2. The resistance of oligos containing natural or modified nucleotides to nucleases is listed in Table I. PS-DNA, MeP-DNA, α -DNA, and 2'-O-alkyl RNA oligos are fully resistant to 5'-exo- and endonucleolytic degradation. The nuclease resistance of oligos with a modified phosphate backbone is dependent on the particular stereoisomer so that snake venom phosphodiesterase, S1, and P1 nucleases digest PS-DNA with the sulfur in either the Sp or Rp position (216) (Fig. 6). All of the modifications listed in Table I decrease the sensitivity to 3'-exonucleolytic attack by at least one order of magnitude, and the PS-DNA and α -DNA modifications confer the strongest protection. For further information on the stability of oligos *in vitro*, in cell culture and in living animals, see 185 and 190–193).

The 5' cap structure protects eukaryotic mRNAs against 5'→3'-exonucleases (226) (Section I,A), apart from binding eIF-4F. This observation has fostered the idea of increasing the half-life of oligos by modifications of the two respective ends (281–284). Even single PS-DNA, PA-DNA, and α -DNA nucleotides or an inverted PO-DNA nucleotide placed at the ends of other oligos (PO-DNA, MeP-DNA, etc.) can serve as “caps” and protect an oligo against exonucleases (192). In another approach aimed at “hiding” the oligo ends, a PO-DNA oligo was extended at either end with a hairpin structure of extreme stability ($T_m = 76.5^\circ\text{C}$). The hairpin, while minimally interfering with hybridization, dramatically increases the stability against phosphodiesterase I (285). Several pendant groups have also been described to function as “caps” that decrease the nuclease sensitivity of oligos (192, 193, 205, 208, 286).

4. RNase H

To study mRNA translation (and other RNA-dependent processes) with oligos, it is often necessary to keep the oligo, the target mRNA, and the resulting hybrid intact. The major threat to the mRNA in the hybrid is posed by RNase H, an enzyme that normally degrades RNA primers bound to DNA during the replication process (287). Although DNA replication is a nuclear event, the presence of RNase H in cytoplasmic extracts, such as the popular rabbit reticulocyte lysate and wheat germ extract for *in vitro* translation, must be considered (288) (Section IV). The cleavage of the mRNA by RNase H is taken advantage of for specific inhibition of gene expression in cultured cells. It even seems that RNase H cleavage is a prerequisite for high efficiency (see Section IV,B). PO-, PS-, and PS₂-DNA oligos form hybrids with RNA similar enough to DNA·RNA hybrids to be recognized by RNase

H (193). Short stretches of only three consecutive PO-, PS-, and PS₂-DNA nucleotides are sufficient for RNase H to cleave the complementary RNA strand (Table I). Efficient cleavage of the target RNA by RNase H can be achieved with oligos of 2'-O-Me RNA containing short splints of PO- or PS-DNA (245, 289). Hybrids between RNA and α -DNA, MeP-DNA, PA-DNA, 2'-F- and 2'-O-alkyl-substituted oligos do not activate RNase H and therefore leave the target RNA intact (Table I).

C. Cellular Uptake

The relative ease of using oligos in cell-free systems contrasts with the hurdles oligos must pass to be effective *in vivo* (cell culture). In this situation, an oligo must reach the target RNA *inside* the cell. It must traverse the cell membrane and find its way to the compartment where it is desired to exert its effect.

The first successful attempts to affect gene expression in cell culture by DNA oligos were reported in 1978 (290). A decade later, studies on how oligos enter into cells were initiated. Progress in this area has been extensively reviewed (283, 291–297) and only a brief summary is presented here.

The most common way of supplying antisense oligos to cells is to simply add them to the culture medium and allow them to be taken up directly by the cells. The introduction of oligos in ways similar to plasmid DNA transfections has been considered to enhance uptake, as has the addition of lipophilic groups directly to the oligos. For an oligo to enter the cell, it must be *actively* transported across the cellular membrane. The kinetic parameters of this transport are summarized in 297. Uptake of PO- and PS-DNA oligos reaches a plateau after 12 hours at 37°C, whereas no uptake occurs at 4°C. PO- or PS-DNA oligo uptake was maximal at 10 μ M. Human HeLa and African green monkey CV1 cells take up oligos with similar efficiency, whereas HeLa S₃ cells do not. The length and the type of the oligo also influence the efficiency. Oligos up to 30 nucleotides in length are readily taken up. The higher efficiency of uptake of PS-DNA oligos over PO-DNA oligos has been attributed to higher nuclease resistance and their nonspecific adherence to cells due to their inherent "stickiness." In addition to enzymatic degradation of the oligo and nonspecific binding to the outside of the cell, problems in developing solid methods for monitoring the uptake has been another reason that reliable information on the actual pathway of oligo uptake into cells has been difficult to obtain.

The uptake of PO- and PS-DNAs presumably occurs by receptor-mediated endocytosis (283, 295, 298–300). Uncharged MeP-DNA oligos, on the other hand, seem to be internalized by pinocytosis (fluid-phase endocytosis) (217, 301). Information on how other types of oligos (such as 2'-O-

alkyl oligos) enter cells (other than by methods described below) is currently not available.

The low efficiency of uptake of oligos from the medium has prompted the search for alternative methods based on procedures for transfection of tissue culture cells. Carriers [liposomes, calcium phosphate, poly(L-lysine) (PLL), and positively charged macromolecules], receptor ligands (transferrin), electroporation, and microinjection have been used. Oligos delivered by liposomes are efficiently taken up into cells by fusion with the cellular membrane and entry via the endocytotic pathway (283, 291). The oligos are also protected from degradation in the culture medium, if enclosed within the lipid vesicle (not the case for the popular "lipofectin" method, in which the oligos adhere to the surface of the vesicle). Low efficiency of enclosure of the oligo (~3%) is still a limiting factor for this type of delivery (302). Coupling the oligo to PLL, an efficient transmembrane carrier, enhances the uptake (292). The combined use of PLL-conjugated oligos and heparin decreases the otherwise toxic effects of PLL on L929 cells. Oligos linked to proteins, which act as carriers (directly or via receptors), also enter cells more readily than free oligos do (303-305).

A different approach to enhance cellular uptake entails the extension of the oligo with hydrophobic tails, some of which are expected to bind to cell surface receptors. Dodecanol, acridine, cholesterol, vitamin E, and other lipids have all been shown to increase the intracellular concentration of oligos to which they have been attached (Fig. 8) (193, 199, 205, 206, 208, 255, 283, 306).

The export of oligos from the endosome to other intracellular compartments is still a poorly understood phenomenon (294, 296, 297). The most undesirable final destination is unfortunately also the most probable: the lysosome, where nucleic acids are normally disposed of by the cell (307). The distribution of oligos within cells also depends on the mode of delivery. In one study (308), it was shown that the efficiency of oligo uptake is a function of the cell type, and that electroporation is consistently more efficient than passive uptake. After electroporation, most of the oligo was located within the nucleus, some was within endocytotic vesicles, and the remainder was in the cytoplasm. In contrast, in nonelectroporated cells most of the oligo was found in small intracytoplasmic vesicles, little was in the cytoplasm, and virtually none was in the nucleus. A similar distribution pattern was also reported for cells microinjected with oligos (309, 310). Consistent with these results, *c-myc* expression decreased far more rapidly in electroporated cells than in cells that passively took up the anti *c-myc* oligo. Electroporation required less than one-fifth of the oligo, which was sufficient to achieve a comparable level of *c-myc* inhibition.

To study mRNA translation in intact cells using antisense oligos, the oligo must reach its target mRNA in the cytoplasm. At present, methods to direct oligos preferentially into this part of the cell while reducing its accumulation within the nucleus are not available. As characterization of the uptake process and development of improved carrier group progress (208), it may become possible to direct oligos to a target RNA within a desired compartment.

D. Effect of Helicases Involved in Translation

Translation, as well as other cellular processes involving nucleic acids, requires helicases to expose otherwise inaccessible bases. RNA unwinding thus allows base recognition by proteins and/or other nucleic acids. The relatively large number of helicases (proven as well as suggested) reflects their involvement in many biological processes and has recently been comprehensively reviewed (249, 311, 312).

The activity of helicases on oligo·RNA hybrids may represent a “physiological reason” that particular oligos can show little or unexpected effects (e.g., modification of the target RNA) in spite of careful experimental design. Translation requires the mRNA to be single-stranded for the codons to be recognized by both initiating and elongating ribosomes. Translation initiation factor eIF-4A (Section II) is the prototype for the DEAD/DEAH-box group of RNA helicases (the name refers to a conserved amino-acid motif). The ATP-dependent unwinding of mRNA by eIF-4A requires eIF-4B (Section I).

Another putative helicase, Ssl2 from *S. cerevisiae* (313–316), has also been implicated in the translation initiation of mRNAs with secondary structures in their 5' UTRs. *In vitro*, eIF-4A/B only unwinds double-stranded stretches shorter than 10 base-pairs (47). Whether the small ribosomal subunit possesses its own helicase activity or whether this can be attributed to eIF-4A/B is not clear. The complete 80-S ribosome, on the other hand, almost invariably melts secondary structures within the ORF (317–320).

The limitations of substrate specificity of the above helicases are not defined. The elongating ribosome can unwind long hybrids between RNA or DNA and the mRNA, whereas eIF-4A/4B has, to our knowledge, only been tested on dsRNA. It is therefore difficult to predict the effects of particular helicases on hybrids between modified oligos and mRNA.

A different helicase that indirectly affects translation was first characterized in *X. laevis* oocytes (321–323), but has subsequently been found in a wide variety of cell types and in a diverse range of species (324). This helicase unwinds RNA·RNA hybrids and simultaneously converts adenosines to inosines by deamination (325). The unwinding/modifying activity is mainly present in the nucleus, but transiently appears in the cytoplasm during maturation.

tion of *X. laevis* oocytes (326). It uses double-stranded RNA longer than 36 nucleotides as substrate (327). The resulting modified mRNA is not translated and rapidly becomes degraded. The appearance and disappearance of basic fibroblast growth factor (bFGF) during maturation of *X. laevis* oocytes is controlled in such a manner (325).

This section attempts to describe important properties of different oligos, identify relevant considerations, and discuss some of the obstacles that (still) exist to their use, primarily *in vivo*. In the next section, we try to illustrate the ways in which antisense oligo approaches may have fruitful applications in several aspects of translation research.

IV. Applications of Antisense Oligonucleotides to the Study of Translation

The models for mechanisms of translation initiation and its regulation, as described in the first two sections, have gained experimental support primarily through: (1) The use of pharmacological inhibitors acting on the ribosomal subunits. (2) Biochemical fractionation, purification, and cloning of translation initiation factors, mainly from rabbit reticulocyte lysate. (3) Genetic approaches, primarily in *S. cerevisiae*, which have confirmed several aspects of previous biochemical studies. More importantly, though, yeast genetics have expanded our knowledge concerning the selection of the initiator AUG, the function of eIF-2, the role of the poly(A)-binding protein, and the identification of new probable initiation factors. (4) Assessment of systematically mutagenized 5' UTRs linked to reporter ORFs in transfected cells and *in vitro*. The characterization of the translation initiation complexes and intermediates is mostly based on density gradient fractionation followed in some cases by RNA and protein analysis.

In this section, we attempt to combine information from the previous three sections and provide an outline of past as well as possible future applications of antisense oligonucleotide approaches to gain further insights into mechanisms of translation and its regulation.

A. Inhibition of Translation *in Vitro*

Antisense techniques were initially applied to mRNAs to identify the protein product from a gene. Annealing of the cDNA to a complex mixture of mRNAs resulted in a loss of the specific protein band after *in vitro* translation (183, 185). This technique, initially called "hybrid arrest," "hybridization arrest," or "translational arrest," has now been replaced by *in vitro* transcription of cDNAs from phage promoter containing plasmid vectors followed by translation *in vitro*. The utility of *chemically synthesized* DNA

oligos to inhibit translation of Rous sarcoma virus RNA in wheat germ extract was demonstrated at the same time as the first antisense experiments using cDNAs (328). These initial results stimulated progress in the development of oligonucleotides, and their application to shutting off gene expression by providing a system for assaying the biological potential of new oligo derivatives. However, the utility of antisense oligos to examine the process of mRNA translation has not yet been fully exploited.

1. RNase-H-INDEPENDENT ARREST OF TRANSLATION *in Vitro*

The strong inhibitory effect seen by DNA-mRNA hybrids were, as the term "translational arrest" indicates, initially thought to occur through physical blockage of the translation machinery. The recognition of the role of RNase H in the degradation of the mRNA that was hybridized to DNA (288, 329, 330) shed a different light on early DNA-oligo-mediated translational inhibition studies, both in rabbit reticulocyte lysate and wheat germ extract (25, 331-333). The utility and the function of RNase-H-mediated inhibition of protein synthesis have recently been reviewed (334) and we concentrate here on RNase-H-independent mechanisms of translational arrest. While some of the results with DNA-oligos could have originated from hybrid arrest of translation, signified by a physical blockage of translation initiation and an intact mRNA, without exclusion of the involvement of RNase H, the mechanistic aspects of these reports must be interpreted with care (25, 332, 333, 335). Antisense DNA can specifically block translation independently from RNase H in the (rare) batches of reticulocyte lysate where the RNase H content is low enough, as demonstrated in a series of well-controlled experiments (317, 318, 336). Inhibition of translation by DNA oligos in wheat germ extracts is, on the other hand, invariably, partly caused by the high content of RNase H.

In contrast to the way in which DNA-like antisense oligos commonly function, MeP-DNA, PA-DNA, and α -DNA oligos inhibit translation in an RNase-H-independent manner (217, 221, 253, 254, 257, 337-342). Unfortunately, the low affinities of these types of oligos for RNA require high concentrations, which frequently cause nonspecific effects on cell-free translation (2500-fold molar excess of α -DNA oligo over target mRNA) (342). Even a small contamination in the oligo preparation can contribute to nonspecific effects when such high concentrations of oligo must be used. Nonspecific (global) effects can result from activation of eIF-2 kinases (Section II), resulting in a rapid shut-off of protein synthesis, modifications of the mRNA other than RNase-H cleavage, or sequestration of translation factors by binding to the oligos. The positive results for α -DNA and MeP-DNA oligos as *specific*

inhibitors of translation required the utilization of an intercalating agent (339, 343), or even UV-induced crosslinking of the oligos to the mRNA (220, 253, 254, 257).

A comparison between antisense DNA and RNA, 25 nucleotides in length and targeted to parts of the 5' UTR of brome mosaic virus RNA, revealed that both types of oligo specifically inhibit translation. Interestingly, the stronger effect was seen with the RNA oligomers. The difference may be explained either by the RNA·RNA duplex being more difficult to unwind, or by insufficient RNase-H content in the particular batch of rabbit reticulocyte lysate to significantly contribute to cleavage of the RNA hybridized to the DNA. However, without mRNA analysis or internal controls for translation, cleavage of the mRNA or nonspecific inhibitory effects cannot be excluded.

Recently, we and others examined the ability of 2'-O-alkyl-modified antisense oligos to specifically arrest translation (344, 344a). When *in vitro*-transcribed mRNAs were cotranslated in rabbit reticulocyte lysate or wheat germ extract, only translation of the mRNA to which a specific purified antisense 2'-O-allyl oligo had been hybridized was inhibited (Fig. 9). Northern analysis of the mRNAs confirmed that the arrest of translation by the 2'-O-allyl oligo was not a result of cleavage of the inhibited mRNA. In contrast, a DNA oligo of the same sequence also inhibited translation, but induced 100% cleavage of the mRNA. Furthermore, when annealed inhibitory oligos were removed from the mRNA by heat denaturation and size separation after translation, the previously inhibited mRNAs could readily be retranslated. Therefore, arrest of translation by mRNA·2'-O-allyl oligo hybrids is specific and occurs independently of RNase-H or modifying activities that would render the targeted mRNA untranslatable (344a). The strong binding of this type of oligo is also manifested by the observation that already a 12-nucleotide-long specific oligo is sufficient to cause close to 100% inhibition, when annealed to the 5' UTR. The length dependence of inhibition has been observed by others for other types of oligos (332, 338) and correlates with the inhibitory effects by *intra*-molecular helices in the 5' UTR (Section I,A).

In short, α -DNA, MeP-DNA, or 2'-O-alkyl antisense oligos can be utilized to induce proper translation arrest. Whereas the first two types must be furnished with intercalating groups (due to their low T_m) to cause inhibition, 2'-O-alkyl oligos bind strongly enough to the mRNA and do not require further modifications (see Section III,A). PA-DNA oligos suffer from considerable nonspecific effects, while DNA oligos can be utilized for translation arrest only in the (rare) batches of rabbit reticulocyte lysate that are devoid of RNase H.

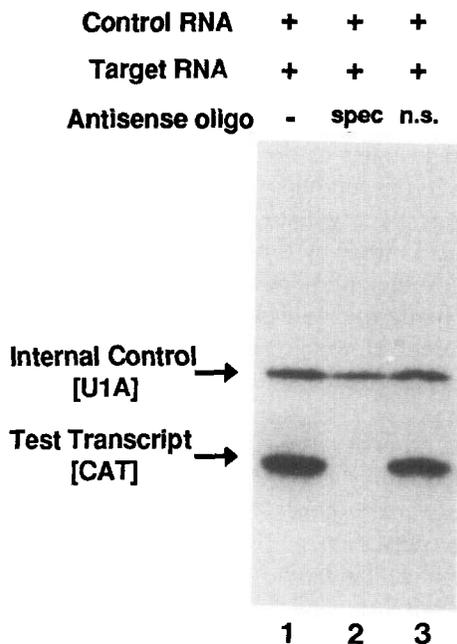


FIG. 9. Target-specific inhibition of translation *in vitro*. Specific (lane 2) and nonspecific (lane 3) 2'-*O*-allyl oligoribonucleotides were annealed to *in vitro*-transcribed mRNAs for CAT (test) and U1A (internal control) prior to cotranslation in reticulocyte lysate. The [³⁵S]methionine-labeled protein products were separated by gel electrophoresis and visualized by fluorography.

2. REGIONS OF THE mRNA SENSITIVE TO TRANSLATION ARREST

How do oligos specifically arrest translation when RNase-H cleavage and mRNA modifications can be excluded? A clue was offered by the demonstration that the entire 5' UTR and a segment extending up to 20–30 nucleotides downstream of the AUG were the only regions of the mRNA sensitive to inhibition by antisense molecules (211, 253, 320, 332, 333, 338, 341, 342, 344a, 345–347) (Fig. 10a).

Studies on the effect of mRNA·oligo hybrids within the major ORF of mRNAs show that positions downstream of the 80-S formation site at the AUG are not sensitive to inhibition by hybrids with antisense oligos (Fig. 10a) (333, 344a, 347). The forceful elongating ribosome removes such blockages (Fig. 10b) (Section III, D), although it appears to stall in front of oligo·mRNA hybrids in the ORF (348) as the 43-S preinitiation complex presumably also does. To our knowledge, there is only one example of complete

arrest of translation elongation by antisense DNA oligos (349). The data presented in this report show the inhibition of translation of the full-length protein and the appearance of a shorter protein product, reminiscent of the pattern observed in 348. The presence of an intact mRNA after translation is of considerable interest, but must be interpreted with care, because it was assessed in a parallel experiment without internal controls.

Like the ORF, oligos annealed to the 3' UTR of an mRNA do not cause antisense inhibition (Fig. 10a) (332, 333, 344a). In contrast to oligos bound to the ORF, the noninhibitory hybrids within the 3' UTR appear not to be dissociated during the translation process (Fig. 10b) (318). This result may have important experimental implications. Oligos with linkers that permit attachment to a solid matrix can be annealed to the 3' UTR and may allow affinity purification of specific messages and their interacting translation complexes, even when bound to the distant 5' UTR (Section IV,C).

As described in Section I, translation initiation involves a number of discrete steps, starting with the binding of eIF-4F to the cap structure of the mRNA (Fig. 1). Since oligos annealed to any part of the 5' UTR and up to 30

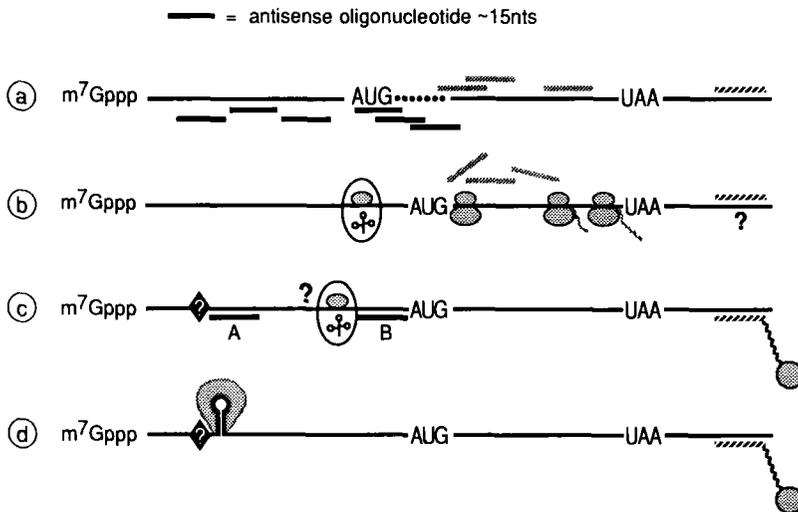


FIG. 10. Antisense-mediated arrest of translation initiation intermediates and their characterization. (a) Regions of the mRNA sensitive to arrest of translation by hybrids with non-RNase-H-activating oligonucleotides. Solid bars represent inhibitory and stippled or hatched bars represent noninhibitory oligonucleotides. (b) Hybrids between mRNA and antisense molecules in the open reading frame are removed by the elongating ribosome. (c) Antisense oligonucleotide-mRNA hybrids in the 5' UTR block translation initiation at different steps (A and B). (c and d) Translation initiation intermediates in the 5' UTR can be isolated by affinity chromatography via an additional end-modified oligonucleotide annealed to the 3' UTR.

nucleotides downstream of the initiator AUG prevent translation, it seems reasonable to assume that more than one of these steps could be affected.

The interaction of the eIF-4E moiety of eIF-4F with the cap of mRNAs in competition with DNA·oligo hybrids in the 5' UTR has been investigated by two different approaches (25, 227, 350). Both show the cap to be accessible to eIF-4E, even if the hybrid starts directly at the first nucleotide of the 5' UTR. If the 3' end of the oligo extends beyond the first nucleotide of the 5' UTR and presumably base-pairs with the m⁷G, binding of eIF-4E is inhibited (350). In reticulocyte lysates, binding of the eIF-4E component of eIF-4F is affected by 2'-O-allyl oligo-mRNA hybrids starting exactly at the first nucleotide of the mRNA (excluding the cap) and extending 15 nucleotides downstream (344a). This finding contrasts with the previous studies using purified factors. While eIF-4E binds directly to the cap *in vitro*, the substantially larger eIF-4F complex very likely constitutes the *functional* entity *in vivo* and *in vitro*. Binding studies to 5' UTR analogs have indeed suggested that up to 35 nucleotides of the 5' UTR may be involved in binding this factor (351).

Oligo-mRNA hybrids further down in the 5' UTR and around the AUG initiation codon most likely inhibit initiation of translation in a manner similar to that of intramolecular helices. In addition to preventing eIF-4F binding to the cap, hybrids may, depending on their position, act by interfering with binding of further initiation factors (eIF-4A/B, eIF-3) (25), the 43-S preinitiation complex, by blocking the preinitiation complex from reaching the AUG or by preventing the 60-S ribosomal subunit from joining the 40-S subunit and the Met-tRNA_i at the AUG.

B. Inhibition of Gene Expression *in Vivo*

1. INTERFERENCE WITH STEPS IN mRNA METABOLISM OTHER THAN TRANSLATION

Numerous reports have described the successful use of oligos for inhibiting the expression of specific genes, mainly with permanent cell lines rather than in primary cultures. This inhibition is mostly due to mRNA degradation, at least in the cases in which the oligos employed are known to stimulate RNase-H activities. The primary cleavages most likely occur in the nucleus, where RNase H and oligo are plentiful (251). This type of inhibition is hallmarked by a decreased level of the targeted mRNA. The primary degradation products can be exceedingly difficult to detect, because they would be unstable within the cellular environment. Cleavage by RNase H, however, clearly indicates that the oligos were bound to the mRNAs. Therefore, other types of oligos that do not elicit an RNase-H response should also be able to bind to (pre-)mRNAs and interfere with steps in mRNA metabo-

lism. Nuclear events, such as splicing and 3'-end processing as well as reverse transcription, which takes place in the cytoplasm, were shown to be affected by intercalator-extended α -DNA and MeP-DNA oligos in Vero (African green monkey kidney) cells as well as by 2'-O-alkyl oligos in *Trypanosoma brucei* (352–356).

2. TRANSLATION ARREST

We have recently tested the potential of 2'-O-allyl oligos to be taken up by, and arrest translation in, human HeLa and murine B6 cells (K. Pantopoulos and M. W. Hentze, unpublished). As determined by indirect fluorescent labeling, the oligos were not efficiently taken up when added to the medium at concentrations up to 20 μ M. Uptake was instead achieved by transfection using a calcium phosphate coprecipitate or lipofectin. In spite of successful internalization of specific and nonspecific oligos, no appreciable difference in the translation of indicator mRNAs could be observed, although the same oligos inhibited translation of the indicator mRNAs *in vitro*. The lack of *in vivo* translation arrest with 2'-O-allyl oligos may relate to a failure to reach the cytoplasmic compartment in sufficient concentration. Alternatively, the target mRNA might be inaccessible to the oligo within the cell, possibly due to interactions with cellular protein(s). Finally, the possibility that the oligo transiently hybridizes with the mRNA but is rapidly dissociated by cellular helicases cannot be excluded.

The successful application of RNase-H activating PS-DNA oligos and their comparison to 2'-O-allyl oligos in repressing expression of SV40 large T antigen (Tag) in CV1 cells and rat fibroblasts was recently reported (251). The authors found that the 2'-O-allyl oligos microinjected into cells failed to affect Tag expression. In contrast, oligos able to activate RNase H caused a dramatic decrease in tag expression. Other results obtained with 2'-O-Me oligos (344) and 2'-O-alkyl oligo variants (245) further support the notion that RNase-H stimulation plays an important role in mediating strong and reproducible inhibition of gene expression *in vivo*.

Several conclusions can be drawn from the application of *doubly* modified non-RNase-H-activating PS-2'-O-Me oligos to inhibit the expression of intercellular adhesion molecule 1 (ICAM-1) in human lung carcinoma A549 cells (357). PS-DNA oligos repressed ICAM-1 expression more efficiently and with less target site-dependence than PS-2'-O-Me oligos. For PS-2'-O-Me oligos, only one of those directed against the 5' UTR repressed ICAM-1 expression. The repression by PS-DNA oligos correlated with a reduction in ICAM-1 mRNA, whereas the 5' UTR PS-2'-O-Me oligo did not affect the mRNA level. Finally, neither type of oligo affected transcription. While the effect of the 5' UTR PS-2'-O-Me oligo is consistent with translational arrest, alternative mechanisms such as inhibition of nucleo-cytoplasmic transport

must be considered, as the RNA analysis was performed on total cellular RNA.

Indirect evidence that RNA·RNA hybrids within the 5' UTR of the mRNA can negatively affect translation *in vivo* comes from experiments with African green monkey kidney TC7 cells, where antisense RNA was coinjected with a reporter plasmid (358). The inhibition of expression of the reporter protein was specific and dose-dependent. Antisense RNA transcribed from another coinjected plasmid also repressed expression efficiently. Furthermore, inhibition of mutant p21 *ras in vivo* with psoralen-linked MeP-DNA oligos indicates that translation can be specifically blocked if the oligo is crosslinked to the target mRNA (339). Direct evidence for translational arrest by mRNA·oligo hybrids *in vivo* comes from experiments with acridine-conjugated α -DNA oligos. The coinjection of target β -globin mRNA and a cap-proximal antisense oligo in *X. laevis* oocytes reduced β -globin synthesis incompletely but reproducibly (235).

C. Possible Future Applications

To give examples in which major open questions about translation and its regulation may profit from antisense oligonucleotide techniques, we discuss briefly the possible applications of antisense technologies to study intermediates of translation initiation and the effects of RNA–protein complexes within the 5' UTR. Applications to many more aspects of translation can easily be envisioned.

1. IDENTIFICATION AND PURIFICATION OF TRANSLATION INITIATION INTERMEDIATES

A ribonucleoprotein particle of roughly the size of the ribosome mediates the complex process of pre-mRNA splicing. An informative strategy for analyzing this particle and for characterizing the functional roles played by each of its subunits has been depletion and purification of individual components from splicing extracts. Using nuclease-resistant oligos from the 2'-*O*-alkyl series with biotin or DNP linkers (Fig. 8), efficient removal and isolation of separate small nuclear RNPs were achieved by hybridization of specific antisense oligos to their RNA components or the pre-mRNA (236, 237). We have recently applied a similar strategy to arrest translation initiation intermediates by forming oligo·mRNA hybrids (Fig. 10c) (Section IV,A). Using streptavidin coupled to paramagnetic beads, these initiation intermediates can be isolated under relatively "gentle" conditions (Fig. 10c). This procedure offers advantages over the "stressful" and lengthy sucrose gradient centrifugation and should furthermore allow a higher degree of biochemical resolution between related complexes (344a). Analogous approaches can also be envisioned to examine mechanistic aspects of internal initiation or reinitiation.

2. ISOLATION OF REGULATORY PROTEINS BINDING TO THE 5' UTR AND THEIR MECHANISM(S) OF TRANSLATIONAL CONTROL

For mRNAs that are translationally regulated through elements located in the 5' UTR, the identification and isolation of possible regulatory binding proteins often constitute the next experimental challenge. By using a biotinylated oligo annealed to the 3' UTR of a translationally regulated mRNA, it is possible to analyze regulatory RNA-protein interactions occurring in the 5' UTR (e.g., the IRE/IRP) of the transcript (H. E. Johansson, N. K. Gray and M. W. Hentze, unpublished) (Fig. 10d). This approach provides an alternative to crosslinking of proteins, and even offers the option of subsequent purification. This may be particularly relevant in cases in which proteins do not crosslink in spite of binding to the RNA (359, 360). Extracts from cells that overexpress the mRNA of interest should allow affinity purification of specific factors binding to the mRNA after the addition of an oligo targeted to the 3' UTR. While many cytoplasmic mRNPs are surprisingly stable and withstand sucrose gradient centrifugation, we know nothing about those that do not. In conjunction with affinity selection of translation initiation intermediates (see above), this approach may further allow us to determine at which step in the initiation process the binding of a regulatory protein to the 5' UTR interferes with translation.

D. Final Remarks

This review focuses, somewhat arbitrarily, on the role of the 5' UTR and largely excludes information concerning the role of the 3' UTR and the poly(A) tail in translation initiation. Nevertheless, the "head end" of the mRNA is clearly a major site of the initiation process and, not surprisingly, for translational regulation. While antisense techniques are currently not part of the standard repertoire of experimental methods in this field, the results described above clearly indicate that, in combination with already established techniques, they may facilitate a much needed clarification of the mechanism of translation initiation and its regulation in coming years.

V. Abbreviations

ALAS	5-Aminolevulinate synthase
bFGF	basic fibroblast growth factor
BiP	Immunoglobulin-binding protein
CAT	chloramphenicol acetyltransferase
DAI	double-stranded RNA-activated inhibitor

eALAS	erythroid-specific form of ALAS
FRP	ferritin repressor protein
GCD	general control derepressible
GCN	general control nonderepressible
GEF	guanine-nucleotide exchange factor
GRP	glucose-regulated protein
hGH	human growth hormone
ICAM	intracellular adhesion molecule
IRE	iron-responsive element
IRE-BP	iron-responsive element-binding protein
IRES	internal ribosomal entry site
IRF	iron regulatory factor
IRP	iron regulatory protein
La	lupus erythematosus antigen
LAP	liver-enriched activating protein
LIP	liver-enriched inhibitory protein
MeP	methyl phosphonate
PA	phosphoramidate
PDGF	platelet-derived growth factor
PE _{st}	phosphotriester
PLL	poly(L-lysine)
PO	phosphodiester
PS	phosphorothioate
PS ₂	phosphorodithioate
Ssl	suppressor of stem-loop
TCE	translational control element
TGF	transforming growth factor

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