The common 5' terminal sequence on trypanosome mRNAs: a target for anti-messenger oligodeoxynucleotides

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SUMMARY

Several mature mRNAs of *Trypanosoma brucei* were previously shown to have a common 5' terminal sequence of 35 nucleotides (nt) encoded by a separate mini-exon. To verify whether all trypanosome mRNAs contain this mini-exon sequence at their 5' end, we have tested oligodeoxynucleotides complementary to different parts of the 35 nt leader sequence for their ability to inhibit translation of total trypanosome mRNA. All oligomers tested inhibited translation of trypanosome mRNAs in a wheat germ extract. They had no effect on translation of Brome mosaic virus mRNA and of a trypanosome mRNA for phosphoglycerate kinase modified to remove the mini-exon sequence. Three different 12mers inhibited translation 35-60%; both the 22- and 34mer inhibited translation 95-100%. Incorporation of amino acids decreased proportionally in all protein bands detected in high resolution polyacrylamide gels. Our results show that all trypanosome mRNAs that yield a product detectable in gel contain a mini-exon sequence. We infer that most, if not all, trypanosome mRNAs contain a 5' terminal mini-exon sequence acquired by discontinuous synthesis.

INTRODUCTION

Trypanosoma brucei, a parasitic protozoan of man and his live-stock in Africa, survives in susceptible hosts through antigenic variation (1). This is achieved by the sequential expression of different Variant-specific Surface Glycoprotein (VSG) genes (reviewed in 2-9), coding for the surface coat (10) which completely covers the trypanosome (11). Analysis of VSG gene transcription has shown that VSG genes are split in an unusual fashion: most of the messenger RNA (mRNA) is specified by a single major exon (12,13); the first 35 nucleotides (nt), however, are encoded in a so-called mini-exon (14-16). The mini-exon was found not only to be present at the 5' end of VSG mRNAs, but quantitative hybridization experiments (17,18) and analysis of several non-identified cDNAs (18,19) and the mRNAs for calmodulin (20), tubulin (21,22) and triosephosphate isomerase (23) showed that the mini-exon may be present on many trypanosome mRNAs. The mini-exon sequence is transcribed from a 1.35 kb unit (17,24) into a small capped (25,26) precursor RNA of 140 nt (27-29), called mini-exon-derived RNA or medRNA (28). The

mini-exon sequence and the 5' cap are transferred to precursor transcripts of various protein coding genes, yielding different mature mRNAs with the same 5' end. The nature of this second step is not yet known. An analogous route for mRNA synthesis is probably used by all other members of the order Kinetoplastida, which includes other major pathogens, like T.cruzi and the Leishmanias (reviewed in ref. 9).

The presence of a common 5' sequence on mRNAs should make the kinetoplastid flagellates exquisitely sensitive to anti-sense RNA or DNA complementary to this sequence. Anti-sense RNA is used by Escherichia coli to regulate the translation of some mRNAs (30-32). Based on this principle, plasmids encoding specific anti-sense RNAs have been constructed (reviewed in refs. 32,33) and used to selectively block gene expression in E.coli (34), Xenopus oocytes (35,36), Drosophila embryos(37) and transfected mammalian cells (38-40). Translation of specific mRNAs can also be inhibited by complementary oligodeoxynucleotides (41-47) and some progress has been made in modifying these oligonucleotides to allow their use on intact cells.

As a first step in the construction of modified oligonucleotides that specifically inhibit protein synthesis in T.brucei and other kinetoplastid flagellates we have tested whether regular oligodeoxynucleotides complementary to part of the mini-exon sequence inhibit protein synthesis directed by trypanosome mRNAs in a wheat germ system. We find complete inhibition with long oligodeoxynucleotides, indicating that all T.brucei mRNAs start with the mini-exon sequence at their 5' termini.

MATERIALS AND METHODS

Trypanosomes

The trypanosome clone 118a (Mitat 1.5a) of T.brucei strain 427 (10) was used for these experiments. Trypanosomes were grown in rats and harvested by exsanguination by cardiac puncture, with citrate serving as the anti-coagulant. Total blood was immediately processed for RNA extraction.

RNA

Total RNA was isolated from trypanosomes by homogenization of total blood in guanidine monothiocyanate followed by direct precipitation of the RNA from guanidinium by LiCl (48). RNA was either stored under ethanol at -20° C or dissolved in sterile water at a concentration of 0,5 µg/µl (see in vitro translation). Brome mosaic virus (BMV) RNA was purchased from Promega Biotec.

Construction of Phosphoglycerate kinase transcription plasmids

The PGK-B and PGK-C genes were isolated from the genomic clone pTgPGK2

(49) and cloned in the pSP65 vector (50). A 1450 bp partial ClaI fragment, which contains the entire PGK-B gene and 30 bp upstream- and downstream sequences, was cloned in the AccI site of pSP65. The resulting plasmid, pSPGK-B, in which the 5' end of the PGK-B gene is located at the sp6 promoter, was used for in vitro transcription of the PGK-B gene. The PGK-C gene was isolated on a 1578 bp EcoRV/HindIII fragment which also contains 60 bp of upstream- and 200 bp of downstream sequences. This fragment was cloned in HindII/HindIII cut pEMBL9 (51) to give p9PGK-C. This plasmid was linearized with BamHI and most of the 5' non-coding sequences were removed by limited Bal 31 digestion. The deleted fragments were excised with HindIII and recloned in HindII/HindIII cut pEMBL9. Following sequence analysis of several clones, one of them was used to reclone the PGK-C gene in pSP65 using BamHI and HindIII. The resulting plasmid, pSPGK-C18, in which 18 bp of 5' non-coding sequence is present, was used for in vitro transcription of the PGK-C gene.

In vitro transcription of phosphoglycerate kinase genes

Conditions for in vitro transcription were essentially as described by Green et al. (52). Reactions in 20 μ l containing 1mM rNTPs, 4 μ g of HindIII linearized DNA, 40 mM Tris-HCl (pH 7.9), 6mM MgCl₂, 60 mM NaCl, 2 mM spermidine, 10 mM DTT, 1.5 U/ μ l RNasin, 1 mg/ml BSA and 20 units SP6 RNA polymerase, were incubated for 60 min at 40°C. RNasin and SP6 were obtained from Promega Biotec.

Translation of mRNAs in a wheat germ extract

The commercial wheat germ extract (Amersham Inc.), which had been pretreated with micrococcal nuclease, was used throughout the experiments. Since optimal concentrations of mRNA and potassium ions vary for different mRNAs in this system, the optimal concentrations were determined for total trypanosome RNA. The standard translation mixtures contained in 30 μ l, 0.5 μ g total RNA, 15 μ l wheat germ lysate, 150 mM K-acetate, a mixture of 19 unlabeled aminoacids (without methionine; Amersham Inc.) at 67 μ M each and 40-50 μ Ci ³⁵[S]-methionine (1 mCi/mmol; Amersham Inc.). Reactions were incubated at 25°C; 1 μ l samples were withdrawn at 0, 15, 30, 60 and 90 min to measure ³⁵S incorporation precipitable by trichloroacetic acid (53).

Some endogenous activity was present in the absence of added RNA (see Figure 3). Addition of trypanosomal or BMV mRNAs resulted in a rapid increase of incorporation of [³⁵S] methionine up to the first 60 min. The rate of incorporation reached a plateau at about 60 min. At this point there was a

standard net stimulation over background (54) of 6-10 fold for trypanosome mRNA and 25-30 fold for BMV mRNA.

Inhibition experiments with oligodeoxynucleotides

Oligodeoxynucleotides used in the present experiments (Figure 1) were prepared by a phosphotriester approach (55), using a fully automated synthesizer (Biosearch, SAM1). Oligomers were purified by G-75 gel filtration chromatography and HPLC (22- and 34mers) or by a two-step HPLC procedure including anion exchange and reverse-phase chromatography (12mers). This was essential since the slightest contamination resulted in non-specific inhibition of aminoacid incorporation with all templates, or prevented maximal inhibitory effects due to size heterogeneity of the oligomers which diminishes the concentration of the full length anti-messenger oligomer. Total RNA (0,5 µg in 1 µl) was incubated in the presence of 0,5 - 8 µl oligodeoxynucleotides, to give a final concentration of 1-40 µM oligomer in the standard incubation mixture (30 µl). Control preparations received an equivalent volume of sterile water. Translation was initiated by the addition of the wheat germ extract mixture and analyzed as described. The inhibitory effect of the oligodeoxynucleotides was stimulated when oligomers and total mRNA were pretreated at 80° C for 2 min and preannealed (2 hrs on ice) with the mRNA before the start of translation (data not shown).

Polyacrylamide gel electrophoresis

Electrophoresis of the wheat germ translation mixture to resolve labeled trypanosome proteins was performed in 12 % linear gradient gels (7-15 % acrylamide). The conditions of electrophoresis, fluorography, geldrying and autoradiography were as described by Ploegh et al. (56).

RESULTS

Kinetics of inhibition by oligodeoxynucleotides complementary to the mini-exon sequence

We have analyzed the selective inhibition of translation of trypanosome mRNAs by sequence-specific oligodeoxynucleotides in wheat germ extract. Figure 1 shows the sequences of the oligomers and their complementary binding sites on the mini-exon sequence of *T.brucei* (17). Figure 2 shows the effect of the oligomers on translation of total trypanosome mRNAs. Inhibition by the oligomers increased with the length of the oligomer chain and oligomer concentration. In contrast a 18mer with no homology to the mini-exon sequence did not inhibit incorporation of [³⁵S]-methionine into trichloroacetic acid precipitable material directed by trypanosome mRNA at similar concentrations

Figure 1. Nucleotide sequence of the mini-exon sequence coding for the 5' 35 nucleotides of mature mRNAs in *T. brucei* (17,23) and the complementary oligodeoxynucleotides used as inhibitors. The underlined bases could form a hair-pin in the mini-exon.

(data not shown; see, however, Figure 3B). Conversely, the translation of BMV mRNA was not inhibited by mini-exon specific oligonucleotides at the same concentrations as those which gave maximal inhibition with trypanosome mRNAs (Figure 2). Only the 22mer and 34mer were able to inhibit translation completely (95-100 %) at concentrations in the range of 15-30 μ M. Similar observations were obtained in a rabbit reticulocyte system, but we refrain

Figure 2. Effect of oligonucleotides on protein synthesis in a wheat germ extract directed by total trypanosome RNA. RNA (0.5 μ g) was translated in a standard mixture of 30 μ l. Reactions were incubated for 60 min at 25°C; 1 μ l aliquots were withdrawn to measure 3 S incorporation. The percentage inhibition was determined after precipitation of proteins with trichloroacetic acid and scintillation counting. Values were corrected for endogenous activity; the standard net stimulation was 6-10 fold for trypanosome mRNAs and 25-30 fold for BMV mRNA. For the 12mers a single, but representative, curve is given (the number of experiments performed with the 12mers were, respectively, 2, 3 and 3); for the 22- and 34mer two independent experiments are shown. See methods for experimental details. The * in the 34mer panel indicates the inhibition of the samples used in Figure 3; Δ gives the values of BMV mRNA, translated in the presence of the oligomers indicated in each panel.

Figure 3. Fluorogram of [^{35}S]-methionine-labeled proteins. The effect of the 34 mer (18 μM) on cell-free translation in wheat germ extract (panel A). The source of mRNA used as a template for protein synthesis is indicated above each lane. Electrophoresis to resolve labeled proteins was performed in 12% linear gradient gels (7-15% acrylamide (56)). See methods for experimental details. The + indicates the presence of the 34mer (see Figure 1) during translation. The arrow indicates the VSG 118 pre-protein. The mRNAs in panel B were translated in the presence of an 18mer (5' CTG ACC AGA AGA AAT CTT 3') with no homology to the mini-exon sequence. The fluorogram is from a separate experiment to that shown in panel A. kDa= kilodaltons.

from presenting these data, as similar observations have independently been made by Walder et al. (57).

Analysis of in vitro synthesized polypeptides

Examination of the total translation products by acrylamide gel electrophoresis showed the synthesis of many trypanosomal proteins with molecular weights ranging from 10,000 to 200,000 (Figure 3A, lane 4; cf ref. 58). Translation of mRNAs, as exemplified most prominently by BMV mRNA (Figure 3A, lane 12), produced incomplete products. This is probably due to premature termination and the release of peptidyl tRNA or to endonucleolytic cleavage of the mRNAs in the wheat germ system (53).

Of the large number of polypeptides synthesized with trypanosome mRNAs

as template, all were found to be proportionally affected over the whole concentration range of oligonucleotide used (22mer, 0-14 μ M; data not shown). The presence of the 34mer at 18 μ M blocked the synthesis of all trypanosomal polypeptides (Figure, 3A lanes 5 and 6 (two independent translations); only the endogenous labeled polypeptides were present (compare with Figure 3A, lane 2) even at exposures of 14 days (data not shown). This exposure time should be sufficient to normally visualize products of rare messages (54). The oligonucleotide concentration did not affect endogenous activity (Figure 3A, lane 2 vs. lane 3). Three types of experiments show that the inhibition of translation is specific for mini-exon containing mRNAs. Firstly, the 34mer has no effect on translation of BMV mRNA (Figure 3A, lane 12 vs lane 13; cf Figure 2). Secondly, the 34mer does not prevent the translation of artificial trypanosome mRNAs of phosphoglycerate kinase genes B and C which lack the mini-exon sequence (Figure 3A, lanes 7-10). Thirdly, there is no effect on the translation products of trypanosome RNA and BMV RNA in the presence of a non-homologous 18 mer (5' CTG ACC AGA AGA AAT CTT 3') at the same concentration as the 34mer (Figure 3B, lanes 1 and 2). Similar results were obtained with phosphoglycerate kinase mRNAs B and C (not shown).

DISCUSSION

Our results show that the translation of the mRNAs in a wheat germ system is efficiently blocked by oligomers complementary to the mini-exon sequence. Analogous results have been obtained in a reticulocyte system by us and by Walder et.al. (57). All mRNAs that give detectable products in these in vitro systems contain the mini-exon sequence. This provides firm backing for our earlier speculation (18) that all trypanosome mRNAs start with the same 35-nt sequence.

The effect of complementary oligodeoxynucleotides on translation of mRNAs is known to be affected by the length of the oligomer, the secondary structure of the target RNA and the position of the target in the mRNA (41-47,60). We also observe that the larger oligomers inhibit trypanosome mRNA translation more effectively than the 12mers. No clear effects were found however, of structure and position of target RNAs: oligomer 1 (see Figure 1) complementary to the very 5' end of trypanosome mRNAs was not clearly more effective than oligomers 2 and 3, even though the latter overlap a potential hairpin structure in the mini-exon sequence (P. Laird and P. Borst, unpublished). Similar observations have been reported for anti-sense RNA (35,36,40 vs. 61). Some progress has been made in modifying oligonucleotides to allow their use

in intact cells, especially to circumvent their degradation by exonucleases. Substituting phosphates for alkylphosphonates make the oligonucleotides more resistant to nucleases (43). In cell culture medium the half life of the molecule is extended from 2 min up to 24 hours (P. Miller, personal communication). Such oligophosphonate oligonucleotides have been successfully used as specific inhibitors of gene expression, both in vitro and in cultured cells (44, 46). An alternative way to prevent the action of exonucleases is to link the end(s) of the molecule to an intercalating agent. If the linker is long enough, the intercalating agent will stack with base pairs in the hybrid, thus providing an additional energy of interaction (62,63). Indeed, acridine-oligonucleotides have been demonstrated to be more efficient translation inhibitors of in vitro gene expression (64,65) than conventional oligonucleotides. For a given hybrid stability this will allow a reduction of the length of the anti-messenger oligonucleotide and, consequently, favour their uptake by living cells. Preliminary results have shown a specific anti-viral effect of such acridine-oligonucleotides on SV40-infected monkey cells ((65), and C.Hélène, personal communication). As trypanosomes use the same mini-exon sequence in most, if not all, mRNAs they should be highly vulnerable to even minor inhibition of ribosome passage over this sequence. We are therefore testing whether chemically modified oligonucleotides can be used to inhibit trypanosome protein synthesis in vitro and whether such oligomers can be made to enter trypanosomes in the bloodstream of infected animals.

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