A rapid *in vitro* method for obtaining RNA accessibility patterns for complementary DNA probes: correlation with an intracellular pattern and known RNA structures

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ABSTRACT

A technique is described to identify the rare sequences within an RNA molecule that are available for efficient interaction with complementary DNA probes: the target RNA is digested by RNase H in the presence of a random pool of complementary DNA fragments generated from the same DNA preparation that was used for target RNA synthesis. The DNA region was amplified by PCR, partially digested with DNase and denatured prior to RNA binding. In the presence of single-stranded DNA fragments the RNA was digested with RNase H such that, on average, each molecule was cut once. Cleavage sites were detected by gel electrophoresis either directly with end-labeled RNA or by primer extension. The pattern of accessible sites on *c-ras* mRNA was determined and compared with the known profile of activity of oligonucleotides found in cells, showing the merit of the method for predicting oligonucleotides which are efficient for *in vivo* antisense targeting. New susceptible sites in the 3'-untranslated region of *c-ras* mRNA were identified. Also, four RNAs were probed to ascertain to what extent structure predicts accessibility: the P4–P6 domain of the *Tetrahymena* group I intron, yeast tRNA^{Asp}, *Escherichia coli* tmRNA and a part of rat 18S rRNA.

INTRODUCTION

Targeting specific mRNAs with antisense oligonucleotides or ribozymes permits alteration of gene expression of potential use for therapeutic purposes and for scientific studies. A reasonable assumption is that oligonucleotide activity in cells is related to accessibility of the targeted mRNA regions. A major problem with antisense approaches is identifying these small mRNA portions (1). The regions containing translation initiation sites are not especially susceptible to antisense oligonucleotides nor are they responsible for the most potent effects. One of the approaches for finding the most active antisense oligonucleotides is to synthesize as many oligonucleotides as possible for different complementary regions of the targeted mRNA and to monitor reduction in gene expression upon introduction of these oligo-

nucleotides into cells (2–5). To reduce the number of oligonucleotides that have to be tested in cells, regions predicted, based on computer modeling or structural probing, to be single-stranded can be targeted (6–8). However, information about the relationship between accessibility and RNA structure is very limited. Single-stranded regions may not be accessible for complementary binding. Double-stranded regions may be available for binding due to strand displacement or even triple helix formation.

A preliminary *in vitro* screening for antisense candidate sites can be employed. There are a number of techniques. Gel shift analysis (9) and translation arrest by RNase H cleavage (10,11) are effective but require the synthesis of large numbers of different oligonucleotides. Binding to immobilized oligonucleotides (12,13) is a clever approach whose applicability to long mRNAs remains to be demonstrated.

Another *in vitro* approach, suggested for both antisense (11–17) and ribozyme (18–21) targeting, involves synthesis of a random pool of DNA oligonucleotides and its hybridization to the target RNA. Accessible sites can be revealed after digestion with *Escherichia coli* RNase H (14–17,21). The sites identified are likely to be similarly susceptible in mammalian cells to endogenous RNase H, which is often responsible for effective antisense oligonucleotide activity (22–25). Because all possible oligonucleotide sequences are present in the random pool, all accessible regions within the RNA domain are revealed providing a number of candidates for testing in cells. A simpler alternative technique using a similar strategy is suggested here. It was used to identify the sites that are accessible to complementary DNA probes in five RNA molecules. One of these RNAs, *c-ras*, was previously studied in cells (4), permitting a direct comparison with intracellular data. Structural information for the other four RNAs allowed assessment of the relationship between structure and accessibility. A tRNA and a rRNA were included in the study because they are abundant molecules in cells, can be targeted non-specifically by antisense oligonucleotides and, perhaps, could be responsible for toxic effects.

MATERIALS AND METHODS

Chemicals and enzymes

Nucleotides, deoxynucleotides and dideoxynucleotides were from Pharmacia (Piscataway, NJ). A Rotiphorese Gel 40 solution of

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acrylamide and *N,N'*-methylene bis-acrylamide was from BioRad (Hercules, CA). Radioactive [³²P]pCp at 3000 Ci/mol and [³²P]ATP at 3200 Ci/mol were from Dupont NEN (Wilmington, DE). Total yeast tRNA, used as a carrier RNA to supplement labeled RNA, was from Sigma (St Louis, MO). Nuclease-free water was from Promega (Madison, WI). Ribonucleases T₁ and U₂ and alkaline phosphatase were from Pharmacia (Piscataway, NJ). Phage T4 polynucleotide kinase, T4 RNA ligase and Vent polymerase were from New England Biolabs (Beverly, MA). RNase H, RQ1 DNase (RNase-free), AMV reverse transcriptase and a T7 *in vitro* transcription kit (RiboMAX™ Large Scale RNA Production System) were from Promega (Madison, WI). Microconcentrators (microcon) were from Amicon Inc. (Beverly, MA).

Preparation of DNA oligonucleotides and RNAs

RNAs were either prepared by *in vitro* transcription (the P4–P6 domain of the group I intron, 18S rRNA and *c-ras* mRNA) or extracted from cells (the yeast tRNA^{Asp} and *E.coli* tmRNA). Using PCR the DNA templates were engineered for *in vitro* transcription. This was done using a transcription kit (Promega). After phenol extraction and recovery of RNA following ethanol precipitation, non-incorporated NTPs were removed using microconcentrators (microcon model 50). Purified yeast tRNA^{Asp} and purified *E.coli* tmRNA were generous gifts of Drs A.T.Dietrich and R.Giege (Strasbourg, France) and Drs H.Himeno and A.Muto (Hiroshima, Japan) respectively. RNAs were aliquoted and stored at –20°C.

The oligodeoxynucleotides were prepared on an Applied Biosystems model 394 synthesizer using the phosphoramidite method. Two synthetic DNA oligonucleotides (5'-TGGTGGAGCTGGCGGGAG-3' and 5'-GGGGCTGATTCTGGATTCTGA-3') were used to amplify by RT-PCR the purified tmRNA from *E.coli*. Two synthetic DNA oligonucleotides (5'-TGAAGTGCATCCATATCAACAGA-3' and 5'-AATTTAATACGACTCACTATAGGGAATTGCGGAAAGGGGT-3') were used to amplify by PCR the P4–P6 sequence of the group I intron from a clone (a generous gift from Dr J.Doudna, Yale University, New Haven, CT) and to insert a T7 promoter at the 5'-end for *in vitro* transcription. Two synthetic DNA oligonucleotides (5'-AATTTAATACGACTCACTATAGGCTGGTTGATCCTGCCA-3' and 5'-GCTGAACGCCCACTTGTCC-3') were used to amplify by PCR the DNA sequence corresponding to rat 18S RNA (the clone containing this sequence was a generous gift from Dr I.G.Wool, University of Chicago, Chicago, IL) and to insert a T7 promoter at the 5'-end for *in vitro* transcription. Two oligonucleotides (5'-TAATACGACTCACTATAGGCTGCATCAATGGAGCACAT-3' and 5'-AAATAACA-TAATTGAGGACCATCAGATAACTGTA-3') were used to amplify by PCR a plasmid containing the *c-ras* sequence [this plasmid was from ATCC (Rockville, MA), no. 41050]. For *c-ras* mRNA mapping nine synthetic DNA oligonucleotides (5'-TCGGCAAGAAAACACGG-3', 5'-TCCTCCTCCCCTGGCAG-3', 5'-GGCAACATGAAGTTAAGGCCC-3', 5'-TCAACATCCACTTGCGC-3', 5'-GCTGTTTGGTGCCCTTATGTGC-3', 5'-GTGAAAGGAGGACGTGTCC-3', 5'-GGGGCAGCTCCTGGAAG-3', 5'-GATAACTGTATTTTGCCAGGTGCA-3' and 5'-AAATAACATAATTGAGGACCATCAGATAACTGTA-3') were synthesized and 5'-end-labeled to initiate reverse transcription.

Labeling at the 5'-end of RNAs (*E.coli* tmRNA and the group I intron) and DNAs was performed with [^γ-³²P]ATP and phage

T4 polynucleotide kinase. The RNAs were previously dephosphorylated using alkaline phosphatase (26). Labeling at the 3'-end of RNAs (*E.coli* tmRNA and yeast tRNA^{Asp}) was done by ligation of [^γ-³²P]pCp using T4 RNA ligase (27). Before any further experiments the RNAs were heated to 75°C for 3 min in the required buffers containing both monovalent and divalent cations, mimicking physiological conditions, and slowly cooled at room temperature for 20 min (renaturation) to try to obtain a homogeneous population of molecules.

Creating a pool of complementary DNAs

Using RQ1 DNase the PCR-generated DNA fragments corresponding to the RNA sequences were digested to obtain a pool of fragments ranging in size from ~10 to 50 nt. Usually 50 µg initial PCR products were digested and recovered after phenol/chloroform extraction of the enzyme followed by ethanol precipitation. To remove DNA fragments shorter than 10 nt centrifugation through microcon 3 was performed. Before mixing with target RNAs the DNA fragments were denatured for 2 min at 90°C.

RNase H digestion of DNA–RNA duplexes and gel electrophoresis

RNA was preincubated for 15 min in the RNase H buffer, digested with RNase H for 1 h at 37°C and then preincubated for 5 min in the same buffer with the pool of DNA fragments. The RNase H buffer contained 40 mM HEPES–KOH, pH 8.0, 1 mM DTT, 10 mM MgCl₂ and 100 mM KCl. Digestion reactions were carried out in a total volume of 10 µl with 1–5 µg RNA (when the RNA was directly labeled unlabeled RNA was added to reach that concentration range), 0.1–0.5 µg DNA fragments and 2 U RNase H. The amount of DNA used for the reactions was variable and titrated to produce RNA molecules that were cut only once on average. Two control RNAs were incubated either with RNase H without DNA fragments or with DNA fragments without RNase H. Cleavage sites were detected by gel electrophoresis either indirectly by analyzing DNA sequence patterns generated by primer extension upon reverse transcription of the modified RNAs or by direct identification of the end-labeled molecules.

Quantitation

Data were analyzed by phosphorimager using ImageQuant software from Molecular Dynamics (Sunnyvale, CA). For all the RNAs analyzed the background present in control lanes was subtracted and the number of RNase H-mediated cuts was normalized to the total amount of radioactivity in the lane. To compare the data obtained on *c-ras* mRNA in cells (4) with the *in vitro* results obtained using this technique the total amount of radioactivity corresponding to the oligonucleotides tested in cells was measured. A fully protected RNA sequence *in vitro* was assumed when there were no differences between the normalized control and reaction lanes. A value of 100% protection was then assigned to this RNA sequence. The relative *in vitro* accessibility of all the other RNA sequences which correspond to the complementary sequences of the oligonucleotides tested in cells was then measured for direct comparison with the *in vivo* data. It ranged from 0 to 100%.

Figure 1. Experimental design for determining the regions of RNAs that are available for complementary DNA fragment binding and RNase H cleavage. PCR-amplified DNA is either transcribed using T7 RNA polymerase or partially digested to 10–50 bp fragments using RQ1 DNase. Next, DNA fragments are denatured by heating, annealed to the RNA and digested with RNase H, with an average of one cut per RNA molecule. Finally, cleavage sites are mapped using primer extension analysis and compared with the sequencing ladder. Alternatively, for short RNAs direct gel analysis of 3′- or 5′-labeled RNAs following RNase H digestion was performed.

RESULTS

Comparison of *in vitro* and intracellular patterns of RNA accessibility

The technique used to map accessible sites in RNAs is described in Materials and Methods and diagrammed in Figure 1. To test the method for its utility in revealing candidate sites for antisense targeting *c-ras* mRNA was analyzed, since there are intracellular data available for comparison (4). A representative electrophoretic pattern is presented in Figure 2, showing the 2310–2510 region of *c-ras* RNA. In other experiments the portions of the coding region (140–340 and 2000–2200) and 3′-untranslated region (UTR) (2100–2960) of *c-ras* mRNA has been mapped (data not shown). The –DNA and –enzyme controls show some bands due to the presence of strong stop signals for reverse transcriptase in the RNA sequence or due to partial RNA degradation. The efficiency of RNA cleavage is seen in the difference in radioactivity between the experimental and control lanes.

Figure 3 compares the intracellular and *in vitro* accessibility profiles of *c-ras* mRNA. The accessibility of regions within the *c-ras* mRNA transcript has been compared with those revealed by the 20 oligonucleotides tested in cells (4). Overall there is a correlation between the intracellular and the *in vitro* data. The accessibility of 13 oligonucleotides out of 20 in cells correlates with the *in vitro* results [oligonucleotides nos 5000, 5076, 5132, 5133, 6722, 6723, 6731, 7847, 7853, 7854, 11447, 11453 and 11455, according to the nomenclature of Monia *et al.* (4)]. For the seven remaining ones no clear correlation has been found. For four of them (11445, 11449, 11151 and 5131) accessibility in cells was much lower than observed using our *in vitro* technique. The last three (6721, 8094 and 11459) had greatly increased accessibility in cells. Using this *in vitro* approach four additional sites within the 3′-UTR of *c-ras* mRNA have been shown to be accessible to a similar or even higher extent than the best oligonucleotide, 5132,

Figure 2. RNase H probing of the *c-ras* mRNA using primer extension. Lanes A, G, C and T are sequencing ladders generated by AMV reverse transcriptase in the presence of ddTTP, ddCTP, ddGTP and ddATP. The experimental lanes (marked +DNA) shows extension products from RNA, that was subjected to RNase H treatment in the presence of complementary DNA fragments. Control lanes (marked C) show extension products of RNA that were subjected to incubation in the absence of complementary DNA fragments (lanes marked –DNA) or enzyme (lanes marked –E). The *in vitro* accessibility of four oligonucleotides, already tested in cells, is emphasized. Chemical instability of the RNA at specific positions or structure-induced reverse transcriptase terminations may account for the presence of bands in all lanes, even in those without DNA fragments. The location of each oligonucleotide is indicated by two arrows and a bar; the first and last nucleotides are also shown.

found in cells. One of them coincides with the position of oligonucleotide 5131, which is not very active in the cell (Fig. 3). Other sites at positions 2252–2277, 2725–2743 and 2791–2805 need to be tested in cells. Our *in vitro* analyses suggest that very few RNA regions are accessible for interaction with complementary DNA probes and the lengths of non-interrupted regions that are cleaved by RNase H are usually <6 nt. It should be pointed out, however, that cleavage fragments do not present the whole picture with regard to accessible regions.

Correlation with structure?

To see if accessibility measured by this *in vitro* method correlates with single-strandedness four different RNA molecules about which structural information is known were analyzed. Figure 4 displays representative probing data obtained on yeast tRNA^{Asp}, the P4–P6 domain of the *Tetrahymena* group I intron, rat 18S rRNA and *E.coli* tmRNA. All the cleavage sites are shown in Figures 5–8. In all cases a few sites are cleaved by RNase H to give products that are not present in the control sample without DNA oligonucleotides (Fig. 4). Notice that the presence of strong, medium and weak bands in Figure 4 are indicated, using a color code, in Figures 5–8. For both yeast tRNA^{Asp} and the P4–P6 domain, for which high resolution structures are available (28,29), the cuts are shown on their three-dimensional structures (Figs 5B and 6B respectively). For yeast tRNA^{Asp} the cuts are scattered over the entire molecule, with some preference for the anticodon branch as compared with the acceptor branch (see

Figure 3. (A) Schematic view of the *c-raf* mRNA sequence (35) showing the location of the antisense oligonucleotide targets. Both 5'- and 3'-untranslated terminal regions (UTR) as well as the coding region are indicated. In order to show the position of all the oligonucleotides the size of the three functional domains are not to scale. (B) Comparison of the *in vitro* and intracellular patterns of DNA oligonucleotide accessibility to *c-raf* mRNA. The accessibility for 20 oligonucleotides is shown. Black bars, intracellular; dashed bars, *in vitro*. Oligonucleotides 5000 and 5076 are complementary to the fragments of mRNA in its coding region, whereas all the others are complementary to RNA sequences in the 3'-UTR and positioned in this figure according to their order within the mRNA sequence (from the 5'-to the 3'-end). The map of the location of these oligonucleotides within the *c-raf* mRNA sequence is shown in Monia *et al.* (4).

Fig. 5B for details). The few cuts seen in the group I ribozyme are in the very 5'-end of the molecule and in the region that is close to the P6-P6a domain. The 5'-end of the molecule is single stranded, but there are several single-stranded domains that are not cleaved by RNase H (e.g. J5/5a and L6b).

For rat 18S rRNA (Fig. 7) the results obtained for only a selected RNA domain are indicated (G1038–U1137). The probing results are indicated on the phylogenetically supported secondary structure (30,31). For simplicity the numbering in Figure 4 corresponds to that of Figure 7. The proposed secondary structure for the selected 18S rRNA domain is made of two stem-loops connected by a single-stranded stretch and a stem. RNase H cleaves both loops A1056–C1064 and U1116–C1119, as well as adjacent to two nucleotides on the 3'-side of both loops, suggesting that the two base pairs located at the top of both stems can be melted by complementary DNA probes. Each of the two main stems has an internal loop. Both sides of the internal loop C1099–A1101, G1132–G1135 are cut by RNase H. However, the other internal loop (G1045–A1051, G1069–G1072) is not accessible. The connecting single-stranded domain C1080–U1089 is also cleaved.

Figure 4 shows some of the cleavage sites on *E. coli* tmRNA and Figure 8 shows the pattern of cuts on a phylogenetically (32–34) and experimentally (34) supported secondary structure model of

Figure 4. *In vitro* RNase H probing of yeast $tRNA^{Asp}$, the P4–P6 domain of the group I intron, rat 18S rRNA and *E. coli* tmRNA. Lanes A_L and G_L are A and G ladders (the RNAs were digested with RNase U_2 and RNase T_1 respectively). Lanes C are control lanes where RNA was subjected to incubation in the absence of complementary DNA fragments (lanes marked –DNA) or enzyme (lanes marked –E). The experimental lanes show the RNase H cleavage patterns of the four RNAs. Varying the amount of complementary DNA fragments in the reaction is indicated. For *E. coli* tmRNA the samples were loaded twice at different times to optimize resolution (long and short migrations). For yeast $tRNA^{Asp}$ the two stars emphasize the difference in migration of RNA fragments which are identical in size. Black bars correspond to cleavage sites and their thickness is proportional to the intensity of the cuts.

the molecule. The molecule was an interesting target since it contains several pseudoknots (PK1–PK4 in Fig. 8). The beginning of the coding region is accessible. Several other regions are also very accessible: part of loops 2 and stems 2 of both PK2 (sequence C183–C199) and PK4 (sequence U287–U300) and to a lesser extent PK1 (sequence A73–C78) and PK3 (sequences A231–A242 and G200–G208). The sequence G321–A326 in H5 (an internal bulge) is also very accessible. There are many weakly accessible regions scattered throughout the whole molecule. Interestingly, the tRNA-like domain of the molecule is not accessible for efficient interaction with complementary DNAs.

DISCUSSION

Advantages and limitations of the method

The technique presented in this paper permits visualization of sites in RNA that are available for antisense interaction *in vitro*. It

Figure 5. RNase H cleavage pattern of yeast tRNA^{Asp} indicated on both the secondary (A) and tertiary (B) structures (28). The colored ribbon in (B) follows the sugar-phosphate backbone. Red, orange and yellow colors indicate strong, medium and weak cleavage sites respectively [arrows in (A) and lines in (B)]. Note that the arrows are pointing to nucleotides, but the cleavage site is in its 5'-side. Note that the last 3' nucleotides, CCA, are missing in the tertiary structure of yeast tRNA^{Asp}.

demonstrates the utility of *in vitro* methods in conjunction with *in vivo* testing for the antisense strategy. An intracellular study of a 1000 nt portion of *c-ras* mRNA identified only one out of 35 oligonucleotides to be complementary to a highly accessible RNA site. Our *in vitro* approach has quickly identified this same site and four additional sites in a slightly smaller portion of the same RNA region. One of these sites is complementary to an oligonucleotide which was shown to be rather inactive in cells (Fig. 3). The activity of the other three antisense oligonucleotides needs further investigation in cells. In order to fully validate the *in vitro* mapping technique more mRNAs need to be tested to obtain reliable statistical data.

It was shown here that instead of using synthetic oligonucleotides to direct RNase H cleavage, fragments generated from cleavage of the template DNA itself can be used. However, it should be taken into consideration that DNase cleavage is not fully random. Because all DNA fragments are generated from exact complementary sequence to mRNA in contrast to the inexact matches of many of the random oligonucleotides, a much lower concentration of DNA can be used, thus avoiding the additional purification step of labile RNA prior to RNase H treatment or reverse transcription. It is quicker than previous methods, does not require synthesis of a large number of oligonucleotides and large RNA molecules can be analyzed. In contrast to the random oligonucleotide approach, the present method uses DNA fragments of variable length, 10–50 nt. This may lead to identification of sites requiring long oligonucleotides and therefore is not desirable for *in vivo* antisense purposes. The number of such undesirable sites is likely to be limited and not be a significant drawback to the technique. This suggests that this method may be a useful first step in quickly finding candidate antisense target sites. One should be aware, however, that since each DNA fragment possesses its own

complement in the DNA pool, complementary DNA strands could re-anneal to each other instead of targeting the RNA. This is unlikely in our probing assay, since there is a large excess of the targeted RNAs as compared with the pool of DNA fragments.

To what extent is knowledge of an RNA structure helpful in predicting accessible sites for antisense targeting?

To address this question, four different RNA structures with limited or extensive structural information were tested. *A priori* it might be expected that single-stranded regions would be more accessible than double-stranded regions for interaction with complementary DNAs. This work shows that the strongest cuts are often located in single-stranded regions (see Figs 5–8 for details). However, there are likely steric limitations to oligonucleotide and RNase H access, making the correlation between single-strandedness and accessibility more subtle. This is clearly illustrated in the case of the group I intron (Fig. 6), for which almost all single-stranded regions are not accessible to complementary oligonucleotides or RNase H cleavage, probably due to the compactness of the structure. Interestingly, accessible regions are not always single stranded. Indeed, complementary DNAs can melt both ends of RNA helices or at internal bulges within helices (there are a few examples in Fig. 7). In the case of *E. coli* tmRNA, loop 2 and stem 2 of several pseudoknots in the current model can be melted by complementary DNAs, due to trapping in an open conformation. Nonetheless, RNase H accessibility may be a guide to molecular breathing, especially on the outside of the molecule. In summary, specific RNA tertiary interactions may (e.g. pseudoknots) or may not (e.g. the tetraloop-receptor interaction) be disrupted by complementary DNA oligonucleotides. Interestingly, for *E. coli* tmRNA a detailed comparison of the accessibility profile with

Figure 6. RNase H cleavage pattern of the secondary (A) and tertiary (B) structures of the P4-P6 group 1 intron mRNA (29). The color code is as in Figure 5.

enzymatic and chemical probing data (34) is possible. Overall there is a correlation between single-strand specific probes (nuclease S₁, lead acetate, imidazole, DMS and CMCT) and accessible regions for antisense targeting, but there are few exceptions (e.g. the more accessible region for RNase H cleavage of the entire tmRNA molecule, C183-C199, is only weakly cut by single-strand-specific probes). Overall the present study illustrates the difficulty of predicting accessible sites from known RNA structures. However, the presence of accessible sites in tRNA and in rRNA means that certain sequences have to be avoided in designing antisense oligonucleotides for mRNAs.

***In vitro* and intracellular probing show few accessible sites**

Despite differences, which might be explained by the presence of proteins and other factors in cells, there is a correlation between *in vitro* and intracellular probing of *c-rat* mRNA. The extent of correlation shows that *in vitro* probing has predictive value. The present analysis further highlights the very small number of accessible sites. The length of non-interrupted accessible regions for RNase H cleavage usually does not exceed 6 nt, but RNase H cleavage products, as already stated previously, do not account for all accessible regions. The absence of long accessible regions in the part of mRNA that was analyzed is probably due to mRNA in general being a very folded molecule. If one takes this view then the surprise is that there are any accessible sites, rather than that there are only a few sites.

What is the nature of these sites? Are they functionally important? Is it possible to find hyperaccessible sites which could be accessible in mRNA regardless of their location? The possibility of common features of highly accessible sites is 'not without interest'.

Figure 7. *In vitro* RNase H cleavage pattern of a selected domain of rat 18S rRNA (region 1137-1038) indicated on a phylogenetically supported secondary structure (30,31). The color code is as in Figure 5.

Figure 8. *In vitro* RNase H cleavage pattern of *E. coli* tmRNA indicated on a current model of secondary structure (32–34). The color code is as in Figure 5.

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