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SnoRNA-guided ribose methylation of rRNA: structural features of the guide RNA duplex influencing the extent of the reaction

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ABSTRACT

Eukaryotic rRNAs contain a large number of ribose-methylated nucleotides of elusive function which are confined to the universally conserved rRNA domains. Ribose methylation of these nucleotides is directed by a large family of small *trans*-acting guide RNAs, called box C/D antisense snoRNAs. Each snoRNA targets precisely one of the nucleotides to be methylated within the pre-rRNA sequence, through transient formation of a 10–21 bp regular RNA duplex around the modification site. In this study we have analyzed how different features of the double-stranded RNA guide structure affect the extent of site-specific ribose methylation, by co-expressing an appropriate RNA substrate and its cognate tailored snoRNA guide in transfected mouse cells. We show that an increased GC content of the duplex can make up for the inhibitory effects of a helix truncation or for the presence of helix irregularities such as a mismatched pair or a bulge nucleotide. However, some helix irregularities dramatically inhibit the reaction and are not offset by further stabilization of the duplex. Overall, the RNA duplex tolerates a much larger degree of irregularity than anticipated, even in the immediate vicinity of the methylation site, which offers new prospects in the search for additional snoRNA guides. Accordingly, a few snoRNA-like sequences of uncertain status detected in the yeast *Saccharomyces cerevisiae* genome now appear as likely *bona fide* ribose methylation guides.

INTRODUCTION

In a variety of cellular processes ranging from ribozyme catalyzed cleavage of RNA to telomerase action (1,2) the specificity of bimolecular recognition events involving RNA is achieved through transient formation of Watson–Crick-type helices. Double-stranded RNA in prokaryotes mediates the control of gene expression by ribonuclease III and gene regulation by antisense RNAs (3). Likewise, formation of transient RNA duplexes plays a key part in the specificity of a host of RNA processing reactions in eukaryotic cells. These include several steps of pre-mNA splicing (4), processing of the 3′-end of histone mRNAs (5), early cleavage of pre-rRNA (6) and various types of RNA editing reactions (7–12). A latest addition to the list is the post-transcriptional modification of rRNA nucleotides in eukaryotic cells, as illustrated by recent small nucleolar (sno)RNA studies (13–22).

Eukaryotic rRNAs undergo two prevalent types of nucleotide modification, methylation (mostly on the ribose) and pseudouridylation. These modifications might affect the three-dimensional folding of rRNA and its association with ribosomal proteins and other ligands, although their actual role in the assembly or function of eukaryotic ribosomes still remains elusive (23). The numbers of modified nucleotides in rRNA exhibit substantial variations among distantly related species, with vertebrates having ~100 ribose-methylated nucleotides and 100 pseudouridines, i.e. about twice as many as *Saccharomyces cerevisiae*. Remarkably, in all eukaryote rRNAs nucleotide modifications are exclusively located within the most highly conserved portions of the rRNA sequences, pointing to some important function. In line with this notion, the pattern of modified nucleotides along the rRNA sequence is largely conserved during evolution, with most of the nucleotide positions modified in yeast also modified in vertebrates (23). For some time, a puzzling question has been how the nucleotide positions to be modified are accurately selected within the long nascent pre-rRNA sequence, given that the different sites of modification in rRNA do not share any recognizable feature in sequence or secondary structure which could mediate recognition by common ribose methylases and pseudouridine synthases. Recent breakthroughs in the field of snoRNAs have solved the enigma: for both ribose methylation and pseudouridylation of rRNA, selection of the appropriate nucleotides is mediated by a large collection of site-specific *trans*-acting RNA cofactors, which obviates the need for a complex repertoire of rRNA modifying enzymes (see 19–22 for reviews).

Throughout its synthesis and processing pre-rRNA transiently associates with scores of snoRNAs (19,24,25), most of which share in vertebrates the unexpected gene organization and unique biosynthetic pathway first reported for intron-encoded U14 (26). Based on structural features, snoRNAs fall into two major families, box C/D antisense snoRNAs (25) and ACA snoRNAs (27,28). SnoRNAs of the first family contain two short sequence motifs, box C and box D, and 10–21 nt sequences complementary to rRNA (19,25). Box C (5′-PuUGAUGA-3′) and box D (5′-CUGA-3′) are also found in U3 snoRNA and are involved in association of nucleolar protein fibrillarin with U3 snoRNP (24). A specific box C/D antisense snoRNA is associated with each methylated nucleotide in rRNA, which precisely targets the position

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to be methylated in pre-rRNA through transient formation of a long duplex structure at the modification site (13–16, see 19,21 for reviews). Likewise, each member of the ACA snoRNA family, defined by the presence of a common 3′-terminal ACA sequence (27,28), guides pseudouridylation at a particular rRNA site through specific base pairing with rRNA, involving in this case two shorter stems around the uridine to be isomerized (17,18, see 20,22 for reviews). Both processes are strikingly related, in that the modification always occurs at a fixed distance from a specific structural feature in the guide snoRNA (13–17). Thus each methylated site is at the same location in the long duplexes, always paired to the fifth nucleotide upstream of box D or its variant version box D′ (13–16,19). The integrity of box D is essential for ribose methylation (15) and shifting box D by insertion or deletion of an adjacent nucleotide displaces the cognate site of ribose methylation by 1 nt, either upstream or downstream (14,15). In the substrate RNA the nucleotides participating in the long duplex with the snoRNA suffice to direct the reaction, suggesting that ribose methylation of specific RNAs, targeted by appropriately tailored snoRNAs, could represent a highly selective tool for altering gene expression at the post-transcriptional level (15). However, while the enzyme which catalyzes methylation remains to be identified, several considerations already stress the need for a better delineation of the RNA duplex features required for the site-specific reaction.

Natural duplexes between endogenous box C/D snoRNAs and their cognate sites of ribose methylation in rRNA have a rather large range of size variation, from 10 to 21 bp (average size 12–13 bp, minimal size 10 bp), and some of them exhibit a helix irregularity (13,14,16). Intriguingly, a previous functional analysis showed that even a moderate change in length and regularity of the RNA duplex at the methylation site could dramatically inhibit the reaction (15). Thus while in mammalian cells transfected with an appropriate snoRNA novel site-specific methylation was efficiently directed to endogenous rRNA by a 16 bp duplex, the reaction was completely abolished by moderate shortening of the duplex to 12 bp or by the presence of a single helix irregularity in the 16 bp duplex.

Identifying the basic constraints acting on elements of the double-stranded RNA guide structure has obvious major implications. In addition to providing the basis for an improved design of tailored snoRNAs when using site-specific ribose methylation *in vivo* as a tool, this information will ultimately prove essential for dissecting the enzymology of the process, once the methylating enzyme and other protein cofactors of the reaction are identified. Delineating these constraints should also help to identify new guide snoRNAs by sequence searches, as already achieved for many members of this snoRNA family (13,25,29,30).

In this study we have extensively analyzed the role of various structural parameters of the guide duplex, such as length, GC content and presence of irregularities in the RNA double helix, and evaluated their combined influence on the extent of site-specific ribose methylation, using an *in vivo* assay in transfected mouse cells. We show that the RNA duplex can tolerate a larger degree of irregularity in the immediate vicinity of the methylation site than anticipated, provided the substrate–guide interaction is further stabilized in the distal portion of the duplex.

MATERIALS AND METHODS

Unless otherwise noted, all techniques for cloning and manipulating nucleic acids were performed according to standard protocols (31).

Ribosomal RNA minigenes

Mouse rRNA minigene pW contains the RNA polymerase I promoter and terminator and terminal nucleotides of the 5′ and 3′ ETS of pre-rRNA, together with a fragment of the chloramphenicol acetyltransferase gene (*CAT*) (denoted by filled boxes in Fig. 1a) serving as reporter sequence for the minigene transcripts (32). Minigene pWMe was derived from pW by inserting the synthetic 65 bp DNA sequence denoted in Figure 1a at the *Hpa*I and *Eco*RV restriction sites of the pW polylinker.

Constructs expressing snoRNA mutants

All mutants were derived from a previously described plasmid construct (33). The plasmid expressing gWMe guide snoRNA (Fig. 1c) under control of the strong constitutive CMV (cytomegalovirus) promoter in transfected mouse cells was obtained by inserting the *Bam*HI–*Eco*RI fragment of mouse genomic DNA spanning intron 11 and the U20 snoRNA coding sequence (33) into the PRCEN1 eukaryotic vector.

Mutagenesis, transfection and Northern blot analyses

The rRNA minigene and mutant U20 snoRNA sequences were altered by PCR-directed site-specific mutagenesis and all mutations confirmed by DNA sequencing. Mouse L929 cells were transfected by the DEAE–dextran method and routinely assayed 72 h after transfection as described (32). The cell culture was submitted to a 2 h treatment with a low concentration of actinomycin D (0.08 µg/ml) 24 h before cell recovery to stimulate transcription of rRNA minigenes (34). Total cell RNA was isolated by the guanidinium thiocyanate method (35), freed from DNA contaminants (32) and analyzed by electrophoresis on 6 or 8% acrylamide/7 M urea gels. Electrotransfer was performed onto nylon membranes (Amersham), followed by UV light irradiation of the membranes (Hybond-N for snoRNA expression assays, Hybond-N^+ for ribose methylation assays). Northern blot hybridizations were carried out with 5′-^{32}P-labeled oligodeoxynucleotide probes, through a 3 h incubation in 5× SSPE, 1% SDS, 5× Denhardt's, 150 µg/ml tRNA, at a temperature of 15–18°C below the theoretical T_m of the hybrid. Membranes were washed twice with 0.1× SSPE, 0.1% SDS before autoradiography. Intensities of radioactive bands were measured in a Phosphorimager. Each co-transfection was repeated, giving rise to essentially identical results. The snoRNA guide and RNA substrate content of all batches of transfected cells was systematically assayed to ensure that changes in methylation degree were not correlated with major fluctuations in the cellular abundance of the two RNAs. Note that the guide snoRNA was usually detected as a band doublet, with the presence of a 3 nt shorter processing product, the relative abundance of which varied among experiments.

Detection of ribose-methylated nucleotides

Ribose methylation of the target guanosine located immediately downstream of the long G-lacking tract in the minigene transcript (Fig. 1a) was assayed as follows. Total cellular RNA was completely digested with RNase T1 through two successive enzyme treatments (20 min at 37°C and 10 min at 65°C) separated by a heat denaturation step as described (15). Aliquots (20 µg) of the RNase T1 digest were analyzed by electrophoresis on an 8% acrylamide/7 M urea gel and assayed by Northern blot hybridization with 5′-^{32}P-labeled oligodeoxynucleotide P.

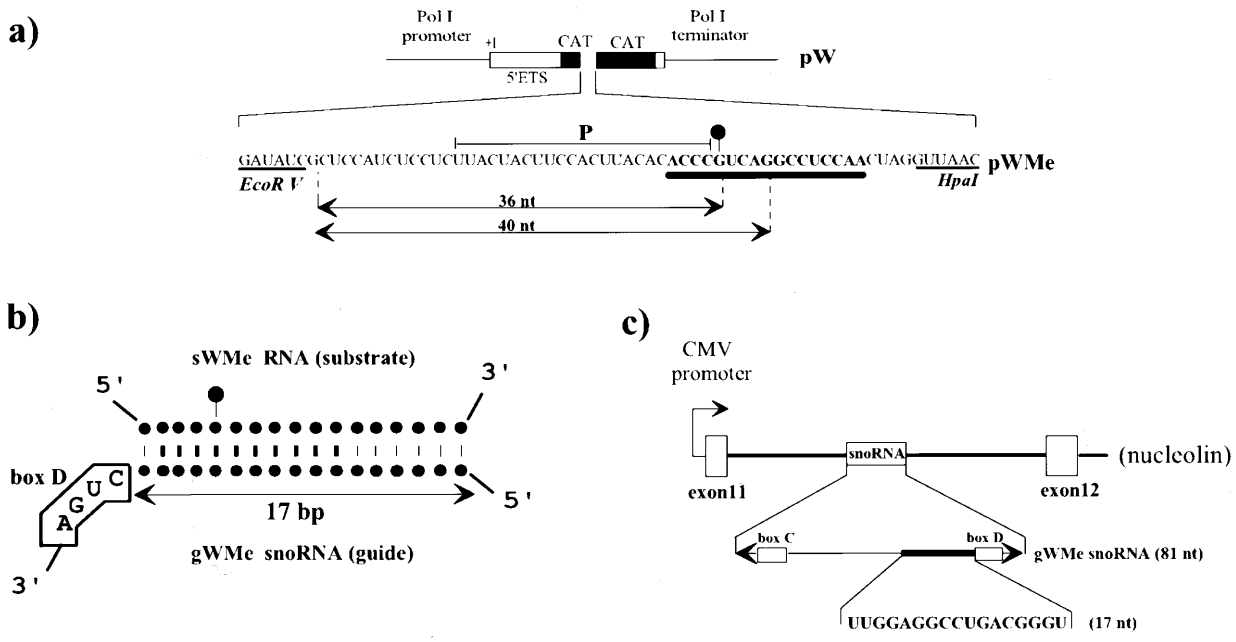

Figure 1. Experimental system for dissecting the role of the guide RNA duplex in site-specific ribose methylation through ectopic expression of an RNA substrate and its cognate guide snoRNA in transfected mouse cells. (**a**) The RNA substrate. The model substrate, sWMe, was expressed by mouse rRNA minigene pWMe (32) under control of the RNA polymerase I promoter and terminator, terminal nucleotides of the 5′ and 3′ ETS regions of the mouse rRNA transcription unit (open boxes) and a portion of the chloramphenicol acetyltransferase gene (*CAT*) (filled boxes) carrying a 65 bp synthetic DNA insert. The insert portion of the transcript (its entire sequence is shown) contains the nucleotide targeted for ribose methylation, i.e. a guanosine (filled circle) within a 17 nt sequence (thick underline) complementary to the appropriate region of the co-expressed cognate snoRNA guide (depicted in c). Owing to the introduction of a long G-lacking tract immediately upstream of the guanosine target the degree of ribose methylation was assayed by measuring the relative abundance of two long RNase T1 oligoribonucleotides (double arrows) by Northern hybridization with probe P. (**b**) The substrate RNA–snoRNA guide duplex. Ribose methylation is directed to the substrate position (filled circle) paired to the fifth nucleotide upstream of box D in the snoRNA. The base pairings conserved in the vast majority of natural snoRNA–rRNA duplexes known so far, i.e. substrate nucleotide positions [–3, +6] relative to the methylation site, are denoted by thick bars. (**c**) The snoRNA guide. A U20 snoRNA mutant, gWMe, was expressed from a construct (top line) in which the snoRNA-containing intron and the two flanking exons of the nucleolin gene are transcribed under control of the strong CMV promoter. In the U20 mutant the natural sequence complementary to 18S rRNA (schematized by a bar) has been substituted by a 17 nt sequence tract able to form with the RNA substrate sequence underlined in (a) the canonical guide structure shown in (b).

Ribose methylation at other nucleotide positions was tested by reverse transcription at low dNTP concentration (36) as follows. Five micrograms of total RNA from transfected cells, mixed with 0.1 pmol 5′-^{32}P-labeled gel-purified oligodeoxynucleotide 3′-CAT2 (32), were dried (SpeedVac) and resuspended in 20 µl 1× reverse transcription buffer (Promega). After a heat denaturation step (90°C, 5 min) hybridization was performed at 55°C for 20 min. Primer extension with 10 U AMV reverse transcriptase (Promega) was carried out in parallel on two aliquots (final volume 40 µl) in the presence of either 4 µM or 1 mM dNTPs.

Oligodeoxynucleotides

All oligonucleotides were synthesized by Y.de Préval (LBME, Toulouse) on a PerSeptive Biosystems Expedite apparatus. After 5′-^{32}P-end-labeling labeled oligonucleotides were either directly used as probes for Northern hybridization or submitted to a prior purification by electrophoresis on a 15% acrylamide/7 M urea gel before utilization as reverse transcription primers.

Expression of the transfected gWMe snoRNA guide and all its mutated derivatives, except for g12(8A:U), was monitored with the same oligonucleotide, o3′PWMe (5′-TGCTCTAGACCGT-CAGACCCGTCAGGCCTCCA-3′; the portion matching the snoRNA sequence is underlined). In the case of g12(8A:U) the oligo 5′-CTGGATCAGAAATGTCATATC-3′ was used.

All minigene transcripts were assayed by Northern hybridization with oligonucleotide probe P (see Fig. 1a for sequence), except for mutant s12(8A:U), which was probed with the oligo 5′-CATTTGTGTAAGTGGAAGTAGTAAG-3′.

The ribose methylation status of portions of *S.cerevisiae* 25S rRNA was tested by reverse transcription at low dNTP concentration with the following primers: o26S-Z5 (5′-CGTTAATCCATTCATG-CGCGTC-3′); o26S-Z2 (5′-TGGTTCGATTAGTCTTTCGC-CC-3′); o26S-Z8 (5′-GTGGGAGATACAGAGAAGTG-3′); o26S-Z3 (5′-CCATTGTAAGTAGTCATCC-3′).

RESULTS

An *in vivo* assay for dissecting the ribose methylation guide duplex

Ribose methylation can be directed to a novel nucleotide position in endogenous rRNA by expressing a transfected box C/D antisense snoRNA containing, immediately upstream of its box D motif, the appropriate sequence complementary to the rRNA target site (15). Conversely, the site-specific reaction is not dependent upon a complex structural organization of the nascent pre-rRNA. It can also be directed to a transcript ectopically expressed from a rRNA minigene by RNA polymerase I transcription, provided the transcript carries the proper sequence complementary to a co-transfected snoRNA guide (15), thus providing an *in vivo* system for analyzing site-specific ribose methylation. The present study, performed in mouse cells co-transfected with the two constructs shown in Figure 1, relies on a straightforward and sensitive assay for

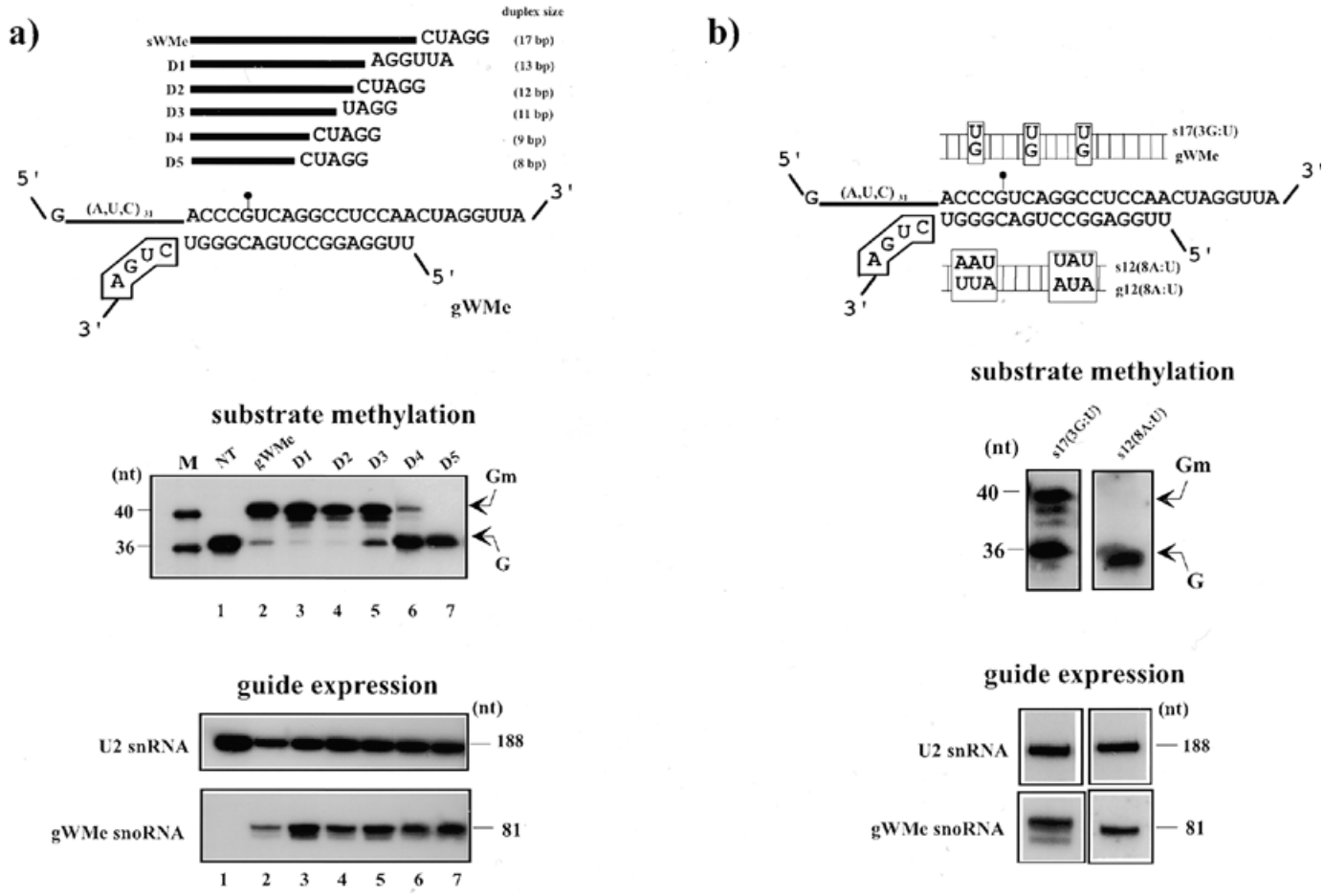

Figure 2. Influence of length and GC content of the RNA guide duplex on the degree of ribose methylation. (**a**) Reduction of the length of duplex extending from box D. (Top) Deleted RNA substrates, derived from sWMe, co-expressed with gWMe snoRNA (the sequence complementary to gWMe remaining in the substrate mutant is represented by a bar). (Middle) Northern hybridization with probe P (see Fig. 1) of a T1 RNase digest of total RNA from cells expressing gWMe snoRNA guide and partially deleted RNA substrates (lane NT, control cells not transfected with gWMe; lane M, size marker). (Bottom) Control. Expression of gWMe snoRNA guide, assayed by Northern hybridization, in the different batches of transfected cells expressing deleted RNA substrates (U2 snRNA served as an internal reference to normalize the gWMe snoRNA signal in different lanes). (**b**) Introduction of thermodynamically less stable base pairings in the RNA duplex. (Top) Structure of the two mutated substrate RNAs and cognate guide snoRNAs. Substrate RNA 17(3G:U), which carried three C→U mutations as compared to sWMe, was coexpressed with gWMe (the location of the three G:U pairs in the corresponding 17 bp duplex is shown). Substrate 12(8A:U), which carried six (G or C→A or U) mutations as compared to the D2 deleted form of sWMe depicted in (a), was co-expressed with an appropriate snoRNA guide to form a 12 bp duplex containing 8 A:U pairs. (Middle and bottom) Substrate methylation assay and expression of the snoRNA guide, as in (a).

quantifying the extent of site-specific methylation which circumvents the severe limitations presently associated with detection of this nucleotide modification (37). Given that the phosphodiester bond immediately downstream of a 2′-*O*-ribose-methylated guanosine is resistant to RNase T1, a guanosine was systematically selected as target nucleotide in all experiments described below. Moreover, the sequence of the RNA substrate upstream of the target guanosine was chosen so as to allow for the appearance of two long oligoribonucleotides after RNase T1 digestion, which can be readily titrated by Northern blot hybridization. As schematized (Fig. 1a), the two radioactive bands reflecting the lack or presence of ribose methylation on the target nucleotide were 36 and 40 nt long respectively.

When the two transfected RNAs were able to form a 17 bp long canonical duplex the target nucleotide in the RNA substrate was ribose methylated to >90% (Fig. 2a, lane 2, gWMe), as judged from the relative intensities of the 36 and 40 nt bands, in line with a previous report (15). We therefore set out to analyze the effect on the reaction of alterations in the RNA duplex, namely reducing its size, modifying its GC content and introducing non-canonical base appositions and bulged nucleotides.

Minimal length of the guide RNA duplex

In a first set of experiments we altered the sequence of the rRNA minigene transcript downstream of the selected guanosine target so as to reduce the length of its sequence complementary to the co-transfected snoRNA guide, gWMe (Fig. 2a). As shown in Figure 2a (middle, lanes 3 and 4), when the size of the duplex was reduced to 13 (mutant D1) or even to 12 bp (mutant D2) while leaving the box D-proximal portion of the complementary sequence intact the extent of methylation was not decreased; actually, the relative level of 36 nt product was even slightly lower than in the control (lane 2), probably due to less efficient transfection with gWMe, reflected in a strongly reduced level of guide expression in that particular experiment (Fig. 2a, bottom). A further shortening of the duplex to 11 bp (mutant D3) resulted in a significant decrease, but the degree of methylation still

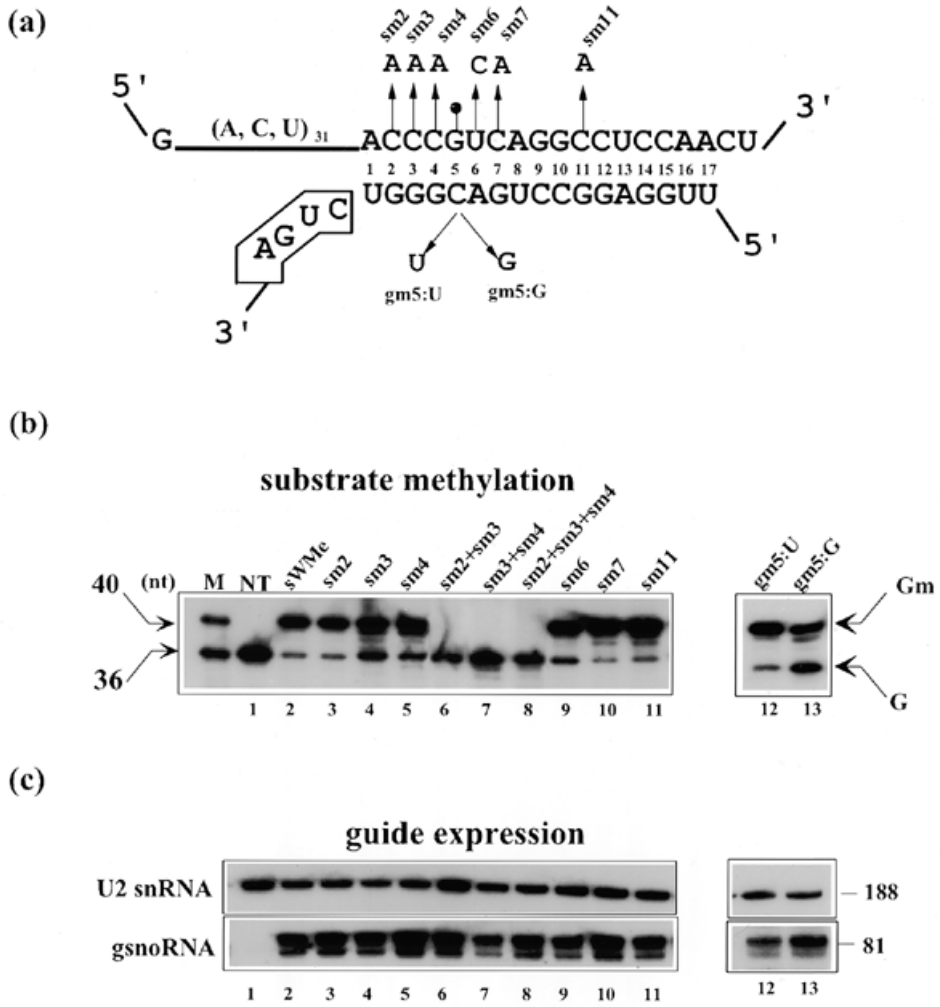

Figure 3. Effect of various non-canonical base pairs in the RNA duplex. (**a**) Location and nature of the mutations. (**b**) Ribose methylation of the G target assayed by Northern hybridization (as in Fig. 2) when the corresponding point mutation (m*i*) or combination of point mutations (m*i*+m*j*) is introduced in the substrate or in the guide. (**c**) Control. Expression of the corresponding snoRNA guide (lanes 2–11, gWMe; lane12, gm5:U; lane 13, gm5:G) measured by Northern hybridization in the different batches of transfected cells.

remained very high, at 75–80%. A further truncation of the duplex preserving only the nine box D-proximal base pairs (mutant D4) dramatically depressed the reaction. However, a significant degree of ribose methylation was still detectable under these conditions, corresponding to ~20% of the level observed for the original 17 bp duplex. Finally, the reaction was completely abolished when the duplex was shortened to 8 bp (mutant D5).

The GC content of the duplex strongly influences the reaction

The finding that the 9 bp long duplex involving mutant D4 was still functional to a very substantial extent was intriguing, given that the shortest natural guide duplexes detected so far are 10 bp long (19). Interestingly, the D4 duplex is GC rich (only three AU base pairs), suggesting that thermodynamic stability of the double-stranded RNA structure, rather than mere length, might represent a critical parameter in the reaction. To directly test this possibility we performed another set of co-transfections with different pairs of minigene transcripts and snoRNA guides, selected so as to lower the GC content of the guide duplex (Fig. 2b). The duplex involving the D2 mutant, still fully functional at 12 bp (Fig. 2a), was selected as a good indicator. Six point mutations were introduced into the minigene transcript and compensatory nucleotide changes also performed in the co-transfected snoRNA guide so as to replace six GC pairs by AU pairs in the duplex (Fig. 2b). The effect of this replacement was quite dramatic, with the level of methylation dropping to <1% after co-transfection with guide 12(8A:U) and substrate 12(8A:U) mutants.

A non-canonical base apposition is tolerated in a long GC-rich duplex

The 17 bp duplex involving the sWMe minigene transcript (Fig. 2a) is GC rich (only six A:U base pairs). This suggested that it could be much more tolerant to the presence of non-canonical base pairs than the duplex specifying novel methylation on endogenous rRNA (15): that duplex was 16 bp long (in fact 17 bp including the first nucleotide of box D) and contained 10 A:U base pairs. To test this possibility three C→U transitions were introduced together in minigene transcript sWMe, giving rise to mutant s17(3G.U), resulting in the appearance of three G.U wobbles in the duplex formed with snoRNA gWMe. This alteration resulted

in only a 50% reduction in the degree of methylation (Fig. 2b). This effect was relatively mild as compared with the dramatic inhibition observed for site-specific methylation of an endogenous rRNA target after introduction of a single G.U in the 16–17 bp long AU-rich duplex (15). Meanwhile, introduction of a single G.U at any of the three positions indicated in Figure 2b did not appreciably reduce the degree of methylation (not shown).

In a next step we systematically tested the effects of non-canonical base appositions (not only the less destabilizing G.U wobble) introduced at various positions of the 17 bp long GC-rich duplex (Fig. 3). When tested on novel ribose methylation of endogenous rRNA directed by a 16–17 bp AU-rich duplex (15) such changes had a very dramatic effect, with at least a 100-fold inhibition. In contrast, their impact was very modest on the system of co-transfected RNA substrate and snoRNA guide forming a long GC-rich duplex (Fig. 3b). Thus point mutations in the minigene transcript resulting in the presence of A.G or C.A appositions in the duplex at positions –3 (mutant sm2), +2 (mutant sm7) or +6 (mutant sm11) relative to the guanosine target did not substantially reduce the degree of ribose methylation. Some significant inhibition could be detected when the single non-canonical apposition was introduced in the immediate vicinity of the target nucleotide, either at position –2 (mutant sm3), –1 (mutant sm4) or +1 (mutant sm6). However, even in these cases the degree of ribose methylation of the guanosine remained very high, at >60%. In contrast, introducing two non-canonical base pairs (other than G.U) in the box D-proximal portion of the 17 bp GC-rich duplex completely abolished the reaction (double mutations sm2+sm3 and sm3+sm4).

A non-canonical base pair was also introduced at the target nucleotide position. Since the methylation assay relied on utilization of a guanosine as target, the mutation was carried out this time not on the minigene transcript but on the snoRNA guide. Remarkably, substituting the G.C pair at the target site by a G.U had only a very modest effect on ribose methylation of the guanosine. Even a more destabilizing G.G apposition did not drastically alter the reaction, which reached ~50% of the control level (Fig. 3b). Again, these two results were in marked contrast to what had been observed for the 16–17 bp AU-rich duplex guiding novel methylation in endogenous rRNA (15).

A bulged nucleotide is tolerated at some positions of the snoRNA strand

In a next step we tested the possibility that the 17 bp GC-rich duplex could tolerate the presence of a bulge nucleotide. A series of 1 nt insertions was first performed in the guide snoRNA, at various positions around the nucleotide paired to the target guanosine in the duplex (Fig. 4a). Insertions performed upstream of this nucleotide preserve the 5 nt spacing between box D and the target site and are not expected to shift the methylation. Accordingly, methylation of the target guanosine was tested as above by a Northern blot assay. A 1 nt insertion beyond the seventh nucleotide upstream from box D (mutant g7/8) did not appreciably decrease methylation of the minigene transcript (Fig. 4b). However, moving the 1 nt insertion closer to the methylation site drastically inhibited the reaction, to ~15 and 5% of the control level for mutants g6/7 and g5/6 respectively. When the 1 nt insertion was moved further downstream in the snoRNA sequence the inhibition became even more dramatic. Guanosine methylation was reduced to ~2 and 1% of the control level for insertion mutants g4/5 and g3/4 respectively (Fig. 4b) and became completely undetectable for mutant g2/3, even for prolonged autoradiographic exposures (not shown). In the three latter insertion mutants the spacing between box D and the nucleotide paired to the target guanosine was no longer 5 nt but was 6 nt, which could possibly have shifted the location of ribose methylation in the minigene transcript (14,15). To test this possibility RNA was extracted from transfected cells and analyzed by primer extension with an oligonucleotide specific for the minigene transcript, at a low dNTP concentration to induce pauses at cDNA bases immediately preceding ribose-methylated positions in the template (36). For gWMe and for mutants g7/8 and g6/7 ribose methylation of the target guanosine was readily detected by primer extension, with a band intensity in good agreement with indications from the Northern assay (Fig. 4c), while a 1 nt longer 'stuttered' cDNA band could also be detected, the relative intensity of which varied among experiments, as observed for natural sites of ribose methylation in cellular rRNA (36). Individual methyls may differ widely in the intensity of their effects on reverse transcription (36) and this test is much less sensitive than the Northern blot assay, exclusively devised for the selected guanosine position. Accordingly, the very low levels of ribose methylation observed by Northern assay for g5/6, g4/5 and g3/4 snoRNA guide mutants were not detected by reverse transcription. Nevertheless, the experiment in Figure 4c ruled out the possibility that a substantial extent of ribose methylation was shifted to a vicinal nucleotide of the minigene transcript when an additional nucleotide was inserted between box D and position –5 upstream of box D, as was the case for g2/3, g3/4 and g4/5.

A bulged nucleotide is tolerated at multiple positions of the template strand

A series of 1 nt insertions around the guanosine target was performed in minigene transcript sWMe (Fig. 5a). After co-transfection of mouse cells with gWMe snoRNA and each mutant minigene ribose methylation was assayed as above. As shown by the Northern assay (Fig. 5b), only two of the various insertions substantially affected the degree of methylation of the guanosine, while the four others had very little effect on the reaction. Thus when the additional nucleotide was inserted at any of the three positions immediately upstream of the target, the guanosine in the template was still ribose methylated at >75% (mutants s2/3, s3/4 and s4/5) and the same held true when the insertion was located 3 nt downstream of the guanosine (mutant s7/8). In contrast, an insertion located 2 nt downstream of the guanosine dramatically decreased ribose methylation of this guanosine, to only ~5% of the control level (mutant s6/7), and the reaction was fully blocked when the additional nucleotide was inserted immediately downstream of the guanosine target (mutant s5/6). The reverse transcription assay (Fig. 5c) fully confirmed these results, with a complete disappearance of the low dNTP-dependent band (lanes 2 for s5/6 and s6/7). It also ruled out the possibility that a detectable level of ribose methylation was taking place at novel positions around the guanosine target when expressing any of the above-mentioned mutants. Taken together, all these data show that a surprisingly large number of positions along the GC-rich duplex, including the site of ribose methylation itself, were tolerant to the introduction of a single helix irregularity in the form of either a non-canonical pair or a bulged nucleotide.

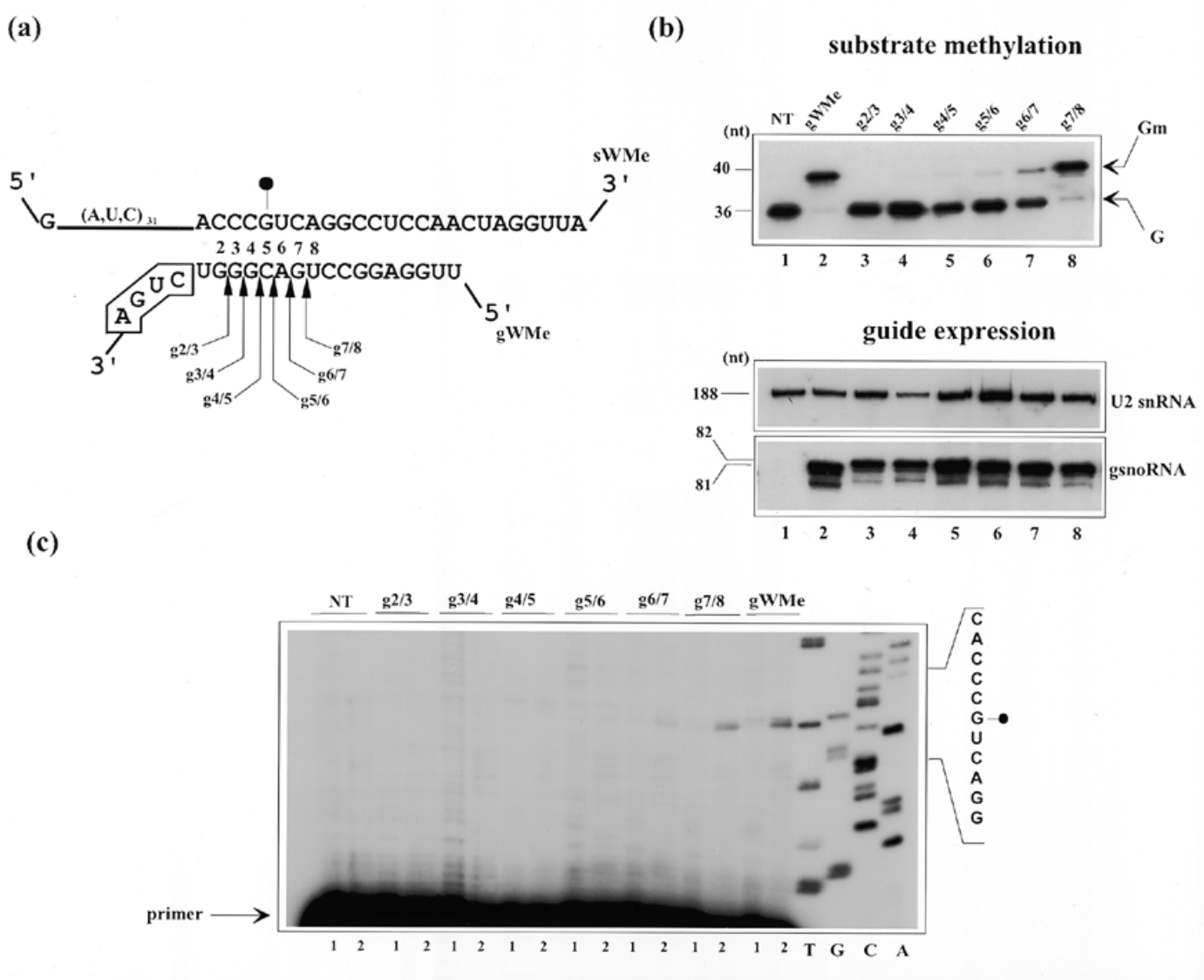

Figure 4. Effect of 1 nt insertions in the snoRNA guide creating bulges in the RNA duplex. (**a**) Location and nature of the different 1 nt insertions. (**b**) Ribose methylation of the G target in the various mutants assayed by Northern hybridization, as in Figure 2 (top). (Bottom) Control. Cellular abundance of the various snoRNA mutants in the different batches of transfected cells, assayed by Northern hybridization. (**c**) Alternative assay of ribose methylation by reverse transcription at low concentrations of dNTPs, which causes concentration-dependent pauses at ribose-methylated nucleotides in the template (37). Primer extensions were performed with the 5′-^{32}P-labeled o3′CAT2 oligonucleotide, either at 4 μM (lanes 2) or at 1 mM (control, lanes 1), using total RNA from cells transfected with the different mutants.

Combined effects of two separate helix irregularities

To further dissect the structural constraints in the duplex we next studied the effect of two separate irregularities on the extent of ribose methylation. In a first step we focused our attention on duplexes in which the target nucleotide was part of a mismatched pair and analyzed the impact of an additional irregularity introduced in the portion of the duplex separating box D and the target site (Fig. 6a). Taken separately, none of such single irregularities dramatically affected the reaction, as detailed above, with effects ranging from no detectable inhibition in most cases to a maximum of ~50% inhibition for a G.G mispairing at the target site. However, the combined effect of two irregularities was dramatic in most cases (Fig. 6b). Thus the reaction was completely blocked when the G.G mispairing at the target site was associated with another mismatch at base pair position 2, 3 or 4 (mutants sm2, sm3 and sm4 respectively) or with an insertion in the minigene transcript creating a bulged nucleotide between base pair positions 2–3, 3–4 or 4–5 (mutants s2/3, s3/4 and s4/5 respectively). However, some of these mutations or insertions in the minigene transcript had a much less drastic effect when associated with a G.U instead of a G.G apposition at the target site. Thus a mismatch at base pair position 2 or a bulge nucleotide between base pairs 2 and 3 still allowed ribose methylation of the guanosine in the minigene transcripts at ~15% when linked to a G.U pair at the target site. Even more striking, insertion of an additional nucleotide immediately upstream of the target position had only a slight detrimental effect in this case: ~50% of the guanosine in the minigene transcript was still methylated (co-transfection with mutants s4/5 and gm5:U, Fig. 6b), instead of ~80–90% when the sole helix irregularity in the duplex was the G.U pair at the target site (Fig. 3, mutant gm5:U). This result was in marked contrast to the complete inhibition observed when the

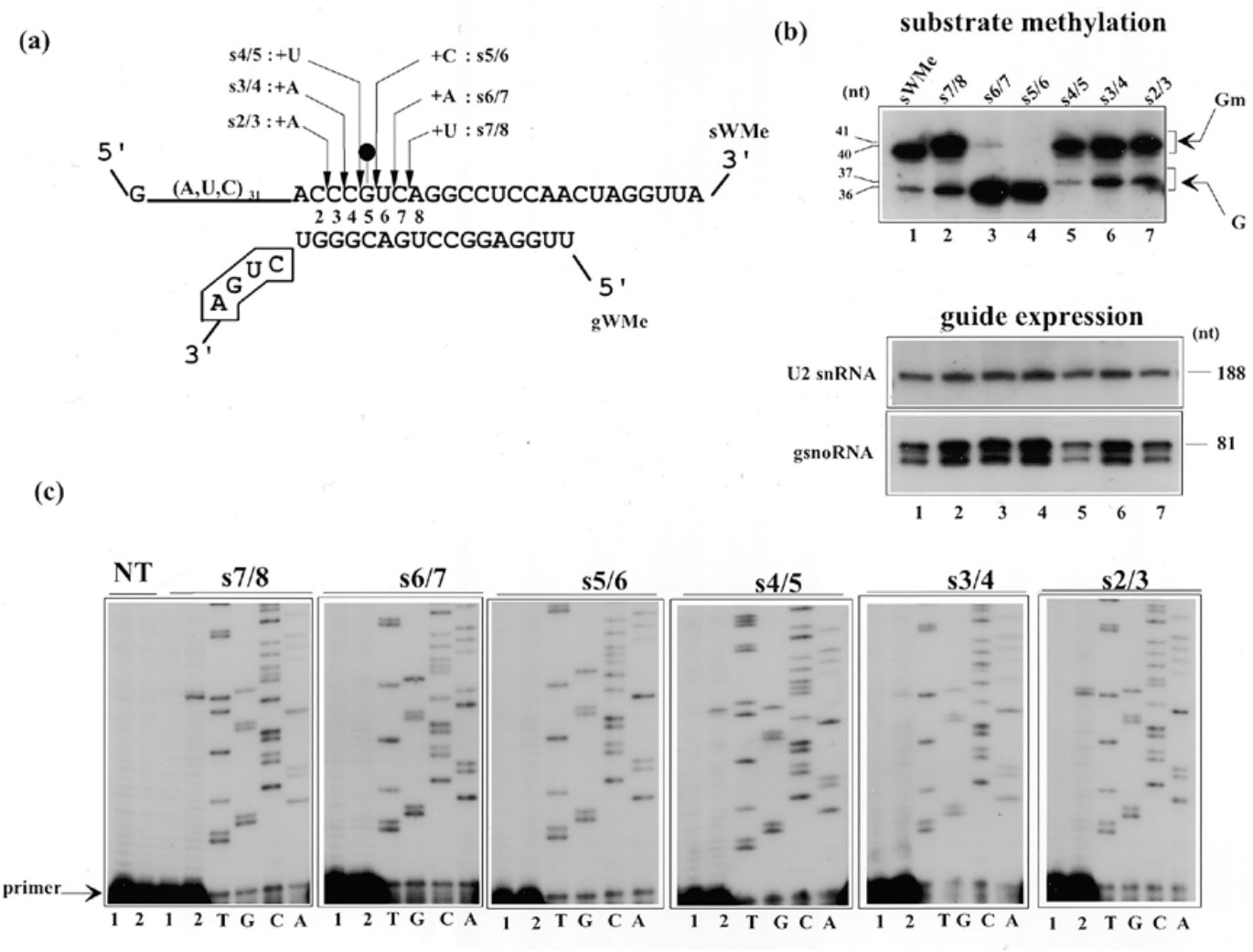

Figure 5. Effect of 1 nt insertions in the substrate creating a bulge in the RNA duplex. (**a**) Location and nature of the different 1 nt insertions. (**b**) Ribose methylation of the G target in the various mutants assayed by Northern hybridization, as in Figure 2 (top). Note that the band reflecting methylation of the guanosine is 41 nt long with all mutants (instead of 40 nt with sWMe), whereas the band reflecting an absence of methylation at this site is 37 nt long for mutants s2/3, s3/4 and s4/5 (but only 36 nt for s5/6, s6/7, s7/8 and sWMe). As a control the cellular abundance of gWMe snoRNA in the different batches of transfected cells was assayed by Northern hybridization (bottom). (**c**) Mapping of ribose-methylated nucleotides by primer extension at low concentrations of dNTPs, as in Figure 4c.

same 1 nt bulge was associated with a G.G mispairing at the target site (lane s4/5 + gm5:G, Fig. 6b). Conversely, mismatches at base pairs 3 or 4 or a bulge nucleotide between base pairs 3 and 4 were completely inhibitory, even when associated with a G.U pair at the target site.

In a second series of experiments we tested the effects of a 1 nt bulge on the snoRNA strand of the duplex (between base pairs 7 and 8), which on its own has no detectable impact on the reaction (Fig. 4b), in association with a variety of point mutations or insertions introduced around the target site in the minigene transcript (Fig. 6c). While a single mismatch at base pair 2, 3, 4 or 6 of the duplex was only slightly inhibitory when the rest of the duplex was a regular helix (Fig. 3b), the combined presence of the two types of helix irregularity dramatically inhibited the reaction (Fig. 6d), particularly when the mismatch was at base pair 3, 4 or 6, for which only ~10% of the guanosine was ribose methylated. Likewise, the introduction of a second 1 nt bulge on the opposite strand of the duplex, between base pairs 2–3, 3–4 or 4–5 was also inhibitory, but to a lesser extent as compared with a mismatch at the same base pair positions.

Identification of new rRNA ribose methylation sites and *bona fide* box C/D antisense snoRNAs in yeast *S.cerevisiae*

Our finding that the RNA guide duplex has a less constrained structure than anticipated opens the way to a more effective search for new guide snoRNAs. To illustrate the point we have focused our attention on a set of eight box C/D snoRNA-like sequences detected by sequence search of the *S.cerevisiae* genome, termed Z2–Z9 (GenBank accession nos Z69294–Z69300 and Z70300 respectively) for which no cognate rRNA ribose methylation site has so far been reported. These sequences either did not match known rRNA ribose methylation sites or exhibited puzzling helix irregularities in their potential duplex with known rRNA ribose methylation sites. Interestingly, 13 ribose methylation sites have not yet been mapped in yeast 25S rRNA (23,38). As shown in Figure 7a, four of these

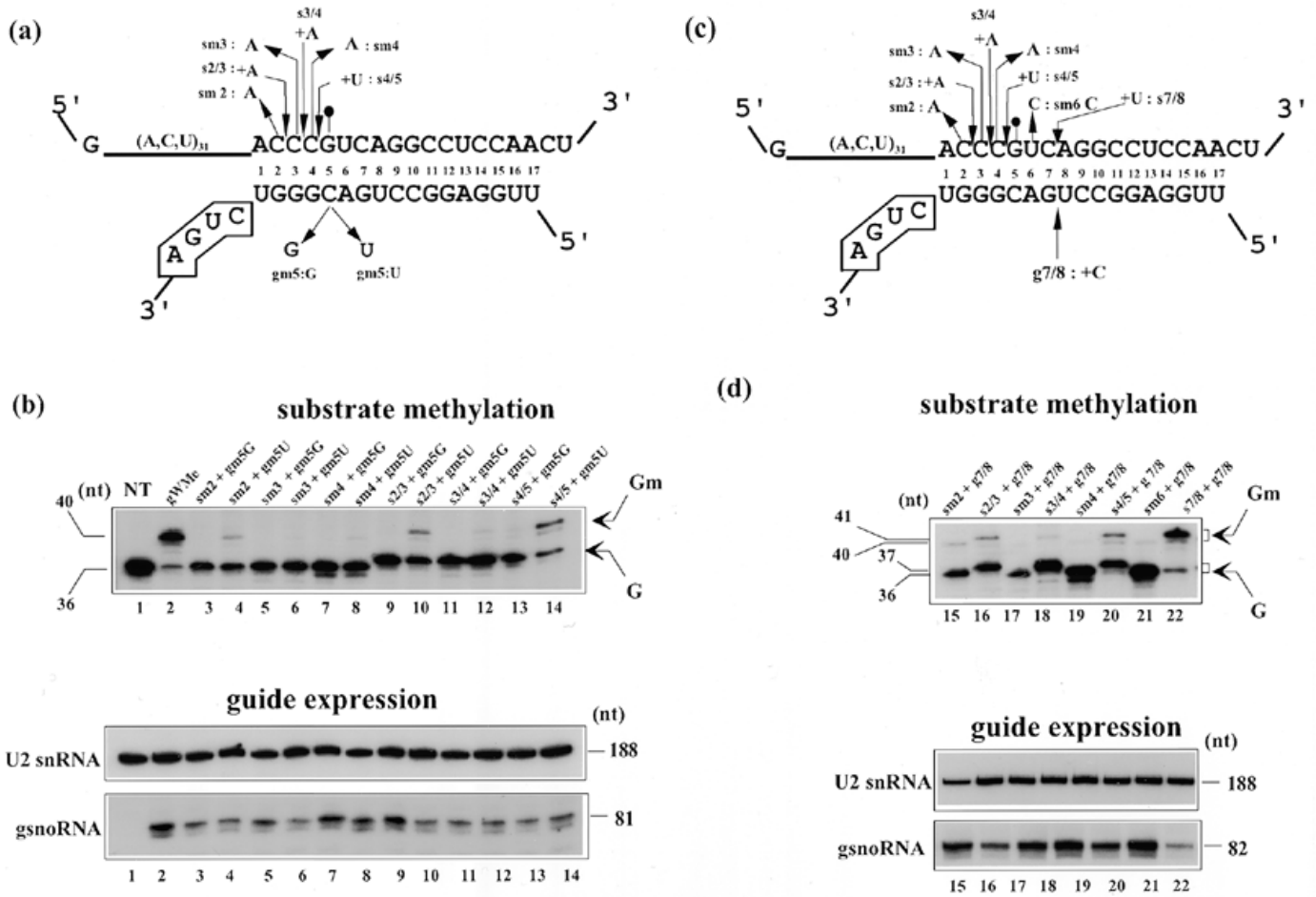

Figure 6. Combined effects of two separate helix irregularities in the RNA duplex. (**a** and **c**) Location and nature of the different single nucleotide changes performed in the guide snoRNA and in the minigene transcript. (a) snoRNA mutants carrying a C→G or a C→U mutation at the fifth position upstream from box D were coexpressed with minigene mutants carrying a single nucleotide change (arrow, with indication of the novel nucleotide). (c) A snoRNA mutant with an additional C inserted between positions –7 and –8 upstream of box D was co-expressed with minigene mutants carrying a single nucleotide change. (**b** and **d**) Ribose methylation of the G target in the various mutants assayed by Northern hybridization, as in Figure 2 (top). (Bottom) Control. Cellular abundance of the snoRNA mutants in the different batches of transfected cells, assayed by Northern hybridization. Note that with insertion mutants s2/3, s3/4, s4/5 and s7/8 the size of the bands reflecting absence or presence of methylation of the guanosine target is 37 and 41 nt respectively.

snoRNA-like sequences, Z2, Z3, Z5 and Z8, are able to form an 11–15 bp long duplex with rRNA outside known ribose methylation sites, in each case through an oligonucleotide sequence immediately followed by a box D (or D′) motif. The duplex involving Z5 contains only Watson–Crick pairings, but the three others display some non-canonical features (thus the 15 bp long Z3 duplex exhibits not only a G.U, at base pair 14, but also a bulged A, between base pairs 2 and 3). However, our present results suggest that none of the irregularities were detrimental to methylation guide function of the duplex. We therefore tested the possibility that the proposed duplexes matched some of the as yet unidentified sites of rRNA ribose methylation. To do so the four corresponding regions of yeast rRNA were directly assayed by reverse transcription at low NTP concentration (Fig. 7c). In each case a new site of ribose methylation was detected, at the expected nucleotide position. This strongly suggests that Z2, Z3, Z5 and Z8 correspond to genuine methylation guide snoRNAs and points to the relevance of the present experimental system for dissecting ribose methylation of endogenous rRNA. Methylation directed by the Z3 duplex (Fig. 7a) has an equivalent in vertebrate 28S rRNA, which involves snoRNA U35 (13,19). We also note that Z7 and Z9 match two known ribose methylation sites in 17S rRNA (23,38), with the corresponding duplexes also likely to be functional despite some helix irregularity (Fig. 7b). Finally, Z4 and Z6 also form regular duplexes (not shown) matching two known ribose methylation sites, both conserved in yeast and vertebrates (23,38), Am27 in yeast 17S rRNA (in vertebrates the cognate snoRNA for this site is U27; 13,19) and Cm2196 in 25S rRNA respectively.

DISCUSSION

The site of rRNA ribose methylation and the efficiency of the reaction are dictated by the combined action of three RNA structural features: (i) the long duplex formed between a box C/D antisense snoRNA and its cognate modification site; (ii) the vicinal box D motif in the snoRNA; (iii) a variant version of box C, box C′ (13–15,39). The relative positioning of the first two elements plays a critical role in defining the precise nucleotide to be ribose methylated in the duplex. The target site is always paired to the fifth nucleotide upstream of box D (or its variant version

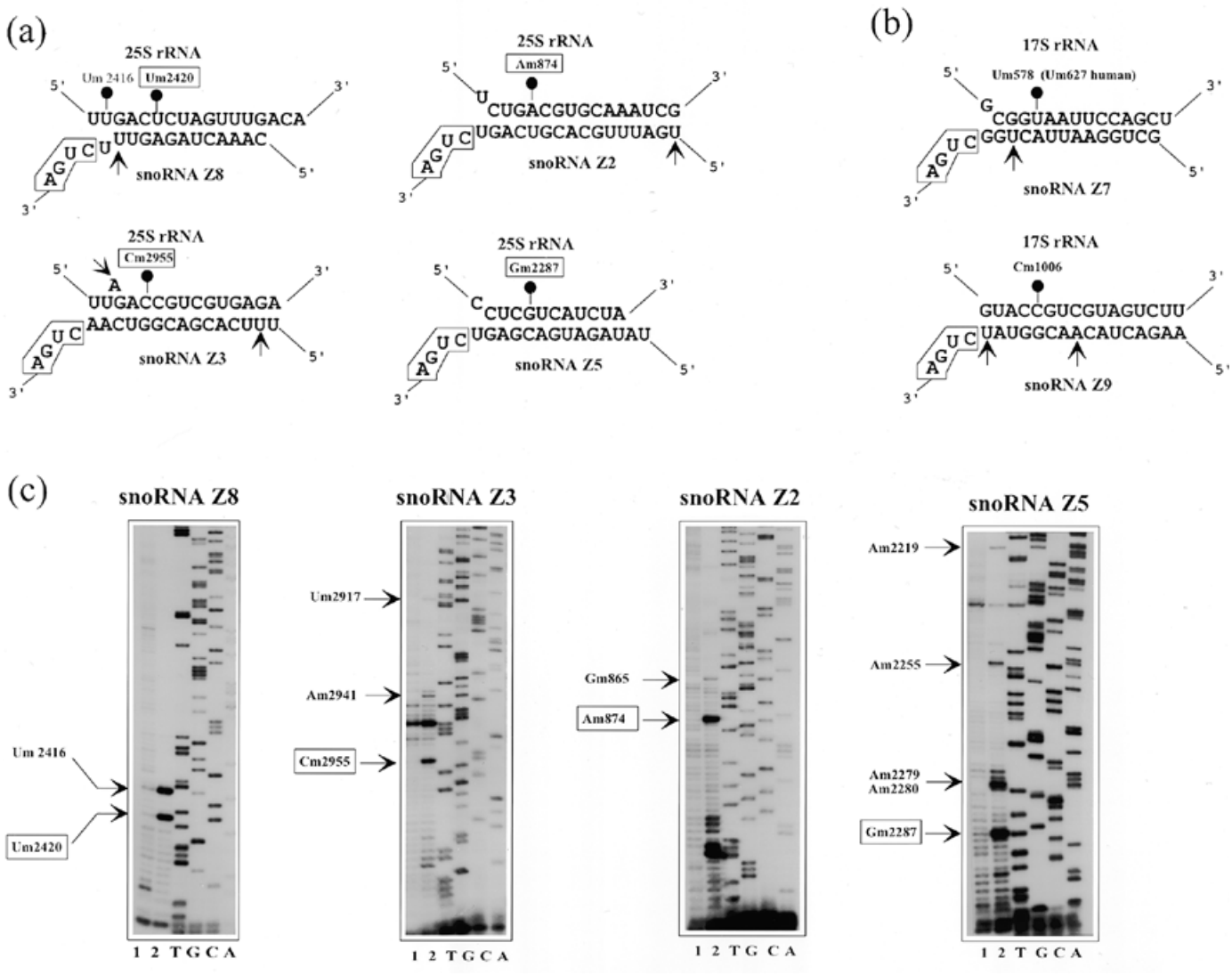

Figure 7. Detection of novel *S.cerevisiae* 25S rRNA ribose-methylated sites and guide snoRNAs. (**a**) Duplexes between presumptive box C/D snoRNAs, identified by a search of the *S.cerevisiae* genome and previously undetected ribose methylation sites in 25S rRNA. The newly identified site, paired to the fifth nucleotide upstream of box D in each duplex, is denoted by a filled circle, with indication of its position along the rRNA sequence (boxed). Non-canonical base pairs and bulged nucleotides are denoted by arrows. (**b**) Duplexes with non-canonical base appositions involving two presumptive box C/D snoRNA sequences and 17S rRNA in *S.cerevisiae* at previously identified rRNA ribose methylation sites (filled boxes). (**c**) Direct identification of predicted previously undetected ribose-methylated nucleotides. Sites of ribose methylation were identified by the appearance of reverse transcription pauses at a low dNTP concentration (lanes 2, primer extension at 4 µM dNTPs; lanes 1, control reaction at 1 mM dNTPs). The newly detected sites are boxed (previously identified ones located in their vicinity and confirmed in this experiment are also denoted).

box D′) in all the complementary sequences between a box C/D antisense snoRNA and a ribose methylation site in rRNA (13,14,19). Accordingly, the methylation in rRNA can be moved in concert with the box D motif in the snoRNA (14,15). The methylase and various protein factors of the reaction have not been identified and the molecular basis of the measuring device involved in site selection remains elusive (37). While an *in vitro* acellular system is not yet available to dissect the reaction, a transfected box C/D snoRNA carrying the appropriate antisense element can direct ribose methylation either to a novel site in endogenous rRNA or to an ectopically expressed short RNA substrate (15). The *in vivo* system involving co-transfection of both the guide and substrate RNAs allows accurate measurement of the effects of alterations of the duplex structure. Utilization of rRNA minigene transcripts as substrates also presents a number of advantages to dissect the reaction in a physiological context. Thus the minigenes are actively transcribed by the cognate RNA polymerase I complex in the nucleolar compartment and their transcripts are faithfully processed for pre-rRNA cleavage (32). Moreover, they undergo natural nucleotide modifications at the proper rRNA sites to a remarkably high extent, provided a minimal segment of rRNA sequence around the modification site is preserved in the transcript, not only for ribose methylation (15) but also for pseudouridylation (17). In both cases the minimal rRNA sequence precisely corresponds to the nucleotides paired to the cognate endogenous guide snoRNA (15,17). In this study we have used minigene transcripts carrying an arbitrary non-rRNA sequence and analyzed its ribose methylation directed by an appropriate co-transfected guide snoRNA. Our results provide new insights into the structural features required in the duplex and

pave the way for a thorough dissection of the double-stranded guide RNA structure, particularly in view of understanding how box D and the duplex cooperate for proper recognition of the 2′-OH group of the target ribose.

Length, GC content and regularity of the RNA duplex

When targeted to a novel site in endogenous rRNA the reaction was dramatically dependent on the length and regularity of the RNA duplex at the methylation site, with a mere 4 bp shortening (from 16 to 12 bp) of the duplex resulting in an ~100-fold decrease in the degree of ribose methylation (15). Likewise, a single non-canonical base pair (a G.U wobble) in the 16 bp duplex abolished the reaction. This observation was intriguing, given that some duplexes between box C/D snoRNAs and natural methylation sites in rRNAs are only 10 bp long (19) and that several of them exhibit non-canonical features in the helix (see Table 1). The present data show that an increase in GC content can fully reverse the functionality of short duplexes, probably because a GC pair provides a higher binding energy than an AU pair. Likewise, the dramatic detrimental effect of helix irregularities at some positions of longer duplexes can be compensated for by extending the box D-distal portion of the duplex. These findings partly account for the above-mentioned paradox, given that the 16 bp duplex active on an endogenous rRNA target and its truncated inactive 12 bp version (15) were both very rich in AU (10 and 8 AU base pairs respectively). However, the large variations in length and stability of the natural snoRNA–rRNA duplexes must also reflect a diversity of constraints acting on the guide structure among the various rRNA methylation sites, with relation to the potential presence of competing intramolecular rRNA base pairings, ribosomal protein interactions and even overlapping snoRNA–rRNA duplexes (13). The shortest natural duplexes are not particularly rich in GC (19) and those with non-canonical pairs (Table 1) are no longer than average. Taken together with our present results, this suggests that the interaction between a snoRNA and its cognate rRNA methylation site could be further stabilized by additional, as yet undefined, base pairings or by protein factors. In this regard the potential involvement of large multiguide complexes ensuring the concerted methylation of multiple sites along the elongating pre-rRNA (37) could also alleviate constraints on the RNA duplex. A truncated ectopically expressed model transcript, devoid of any mature rRNA sequence which could assemble into a complex RNP structure around the target site, therefore presents unique advantages to further analyze the fundamental mechanisms of the reaction.

The location of the CUGA (box D) sequence, about one half turn of the RNA helix from the target nucleotide on the opposite strand, is remarkably close to the ribose methylation site in the three-dimensional structure of the RNA duplex (40) and this basic RNA structural motif could directly affect the geometry of the RNA duplex and play a role of its own in site selection (13,41). Alternatively, the conserved 5 nt spacing might merely reflect specific features of the spatial organization of the snoRNP, which could itself largely determine which ribose is recognized by the methylase. Systematic sequence changes in the duplex resulting in predictable alterations in the geometry of the RNA helix will ultimately provide a valuable framework to test these hypotheses. Pending such analyses, the effects of some of the alterations studied here deserve further comment, even if their significance as to the recognition mechanism involved cannot be fully evaluated for the moment.

Table 1. Helix irregularities in snoRNA–rRNA duplexes at natural ribose methylation sites

Duplex containing a G.U pair
U28: 7, 8 [14bp] (41)
U29: 9 [12 bp] (41)
U30: 2 [12 bp] (41)
U32: 3 [11 bp] (13)
U33: 7 [12 bp] (13)
U35: 7 [14 bp] (13)
U43: 11 [10 bp] (14)
U53: 10 [10 bp] (14)
snR190: 4 [14 bp] (14)
Duplex containing a non-canonical apposition different from a G.U
G.A in U20 (*X.laevis*): 6 [18 bp] (42)
U.U in U49: 6 [13 bp] (14)
G.G in U60: 2 [14 bp] (14)
C.A in U62: 8 [13 bp] (14)
Duplex containing a bulged nucleotide
U29: 8/9 [12 bp] (41)

The relevant base pair position in the duplex is numbered as in Figures 2–6 (the length of the duplex is in brackets and the literature reference in parentheses)

Forbidden and allowed helix irregularities around the target

Surprisingly, a bulged nucleotide can be introduced at a large number of positions without dramatically decreasing the extent of reaction or altering its site specificity in terms of box D spacing. On the substrate strand of the duplex the only bulges which are not tolerated are between base pairs 5–6 and 6–7, immediately downstream of the methylated site. In contrast, a bulge at other positions next to the methylation site has no substantial effect (except when associated with another helix irregularity nearby). Conversely, on the snoRNA strand a bulge is very strongly inhibitory when located at any position between box D and base pair 6 of the duplex, but much less inhibitory when located between base pairs 6–7 and without significant effect beyond base pair 7. In agreement with these observations, the only natural duplex known so far exhibiting a bulge nucleotide, which involves U29 (41), corresponds to the presence of an additional G beyond base pair 7 on the substrate strand. Interestingly, bulges which have little or no detectable effect on the reaction when introduced separately strongly inhibit methylation when associated with different strands of the same duplex, even when separated from each other by a substantial number of Watson–Crick base pairs. Thus in the case of the duplex forming between mutants s2/3 and g7/8 (Fig. 6) the target guanosine, although positioned in the middle of a run of five consecutive canonical base pairs and still paired to the fifth nucleotide upstream of box D, is no longer properly recognized by the methylation apparatus. These alterations delineate a critical subdomain of the RNA double helix, spanning the 7 nt immediately upstream of box D on the snoRNA strand and the 2 nt immediately downstream of the position to be ribose methylated on the substrate strand. They could result from mere steric hindrance brought about by a bulge of the proper contacts of protein components of the methylation apparatus. Alternatively, they could reflect the requirement of the catalytic reaction for a

precise spatial positioning of the 2′-OH group of the target ribose relative to other nucleotides in the guide duplex.

The observation that a single non-canonical base pair is not substantially detrimental, even in the immediate vicinity of the target nucleotide, should also be stressed. Introduction of a single G.U wobble has no detectable effect. Even multiple non-adjacent G.U wobbles are tolerated, with three of them in the same duplex resulting in a mere 50% inhibition of the reaction (Fig. 2b). Remarkably, whereas helix irregularities in natural duplexes known so far correspond mostly to a G.U wobble (Table 1) a more destabilizing base apposition has no, or only a very limited, inhibitory effect in the present system. Thus a particularly destabilizing G.A apposition is tolerated, even adjacent to the target nucleotide (Fig. 3), strongly suggesting that the few reported natural snoRNAs forming duplexes with non-canonical base appositions do represent *bona fide* methylation guides (Table 1, with cases of a G.A and a U.U at base pair 6). In addition, while no natural box C/D antisense snoRNA forming a guide duplex with a wobble at the target position has ever been reported to date, we show that a guanosine paired to a U is still very efficiently ribose methylated (Fig. 3). Moreover, a 1 nt insertion resulting in a duplex with a bulged U immediately upstream of the guanosine in the wobble has only a moderate detrimental effect on the level of methylation (Fig. 6b, lane 14). Even more striking, a target guanosine opposite another guanosine in the long GC-rich duplex is still methylated to a high level.

Implications of a functional duplex with helix irregularities

The complete repertoire of methylation guide snoRNAs is far from being identified, even in the three best studied eukaryotic organisms, *S.cerevisiae*, human and mouse (19). Only half of the ~100 ribose-methylated nucleotides in vertebrate rRNAs have been assigned a cognate guide snoRNA so far. Searches of sequence databases, instrumental in identifying a large fraction of box C/D antisense snoRNAs known to date in vertebrates and yeast (13,25,29,30), have been performed for long rRNA complementary sequences with at most a single G.U pair and devoid of mismatches (13,25,30). Sequence searches taking into account the relaxed constraints on the duplex identified in this work should allow detection of additional methylation guide snoRNAs, particularly in *S.cerevisiae*, as illustrated by the results in Figure 7. Finally, the present data provide the basis for an improved design of tailored guide snoRNAs for *in vivo* site-directed methylation, which should eventually represent a highly specific tool for altering gene expression at the post-transcriptional level.

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