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Substrate specificity of the dsRNA unwinding/modifying activity

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Double-stranded RNA (dsRNA) unwinding/modifying activity, which is present in a wide range of eukaryotic cells, has been previously shown to convert up to 50% of adenosine residues to inosines within intermolecular dsRNA. In the present study, we report that this activity also modifies, though slightly less efficiently, intramolecular double-stranded regions of synthetic RNAs. Our results widen the range of the possible biological substrates for the activity since many stem and loop type RNA secondary structures (intramolecular dsRNA), present in eukaryotic as well as viral transcripts, can potentially serve as substrates. In addition, we have found that the dsRNA unwinding/modifying activity requires a double-stranded region of at least 15–20 base pairs (bp) for substrate recognition. Furthermore, modification efficiency was found to be critically dependent on the length of the double-stranded region; as the size decreased below 100 bp, it dropped precipitously. Our results suggest that efficient modification may occur only with relatively long (>100 bp) dsRNA, perhaps because multiple copies of the enzyme must be bound.

Key words: deaminase/double-stranded RNA/dsRNA modification/dsRNA unwinding/RNA degradation

Introduction

The double-stranded RNA (dsRNA) unwinding/modifying activity (dsRNA unwindase or denaturase), originally discovered in *Xenopus* eggs (Bass and Weintraub, 1987; Rebagliati and Melton, 1987) and subsequently in mammalian cells (Wagner and Nishikura, 1988; Wagner *et al.*, 1990), is a novel biological agent. The activity, localized in the nucleus and present ubiquitously in mammalian tissues and cells (Wagner *et al.*, 1990), converts many adenosine residues of its substrate, dsRNA, to inosine (Bass and Weintraub, 1988; Wagner *et al.*, 1989). The resultant RNA, which contains less stable wobble I–U base pairing in place of Watson–Crick A–U base pairing, becomes increasingly unwound during the reaction, as detected by its sensitivity to single-strand specific ribonucleases (Bass and Weintraub, 1988; Wagner *et al.*, 1989). However, unwinding of the substrate dsRNA is incomplete, and the reaction product remains as a partly denatured dsRNA instead of two completely dissociated strands (Bass and Weintraub, 1988; Wagner *et al.*, 1989). In this sense, the activity is not a typical RNA unwindase or helicase (Bass and Weintraub, 1988; Wagner *et al.*, 1989).

At the moment, the full range of physiological functions of this newly discovered activity is not known. However, several illuminating facts are emerging from the recent studies on its activity. The unwinding/modifying activity may be involved in regulating the fate of dsRNA formed *in vivo*. In maturing *Xenopus* oocytes, it has been suggested that the dsRNA unwinding/modifying activity regulates the rapid degradation of maternal basic fibroblast growth factor (bFGF) mRNAs and antisense transcripts derived from the opposite strand of the bFGF gene (Kimelman and Kirschner, 1989). Aside from its physiological functions, the dsRNA unwinding/modifying activity may also be involved in the modulation of replication and gene expression of certain RNA viruses. In the matrix gene of a defective measles virus, isolated from the brain of a patient with measles inclusion body encephalitis, 50% of the A residues of the minus strand RNA genome were found to be mutated to G (Cattaneo *et al.*, 1988). (Note that I is copied to G in the cDNA formed by reverse transcriptase used for sequencing the viral RNAs.) This biased hypermutation has been suggested to be introduced by the dsRNA unwinding/modifying activity (Lamb and Dreyfuss, 1989; Bass *et al.*, 1989; Cattaneo *et al.*, 1989). Two more examples of highly biased A to G mutation have been found in the hemagglutinin gene of another strain of measles virus isolated from a patient with subacute sclerosing panencephalitis (Cattaneo *et al.*, 1989) and in a defective interfering particle of vesicular stomatitis virus (O'Hara *et al.*, 1984). In addition, the unwinding/modifying activity has been suggested to be responsible for specific A to G base transitions in the human hepatitis delta virus genome occurring during viral replication. In this case, however, the number and locations of A to G mutations are rather restricted, resulting, in one instance, in replacement of a translation termination codon thereby generating the longer form of human hepatitis delta virus antigen (Luo *et al.*, 1990).

Similarly, the range of biological substrate RNA for the dsRNA unwinding/modifying activity is largely unknown. However, there are several clues about potential substrate RNAs emerging from recent studies. The activity is very specific for dsRNA, and it appears to bind preferentially to dsRNAs but not to other nucleic acids such as single-stranded RNA, DNA or tRNA (Bass and Weintraub, 1988; Wagner *et al.*, 1989). Although there is no strict sequence requirement in the substrate dsRNAs, some base preference for the 5′ neighbor of the modified adenosine has been reported (Kimelman and Kirschner, 1989). When the *in vivo* unwound/modified bFGF mRNA was examined after PCR amplification and cDNA cloning, it was found that adenosines having adenosines or uracils as 5′ neighbors were modified more frequently than those having guanosines or cytosines as neighbors (Kimelman and Kirschner, 1989).

Thus, within a given dsRNA with random sequence, adenosine residues in a stretch of consecutive As or an AU-rich region might be preferentially attacked by the dsRNA unwinding/modifying activity (Kimelman and Kirschner, 1989). It has also been shown that intermolecular dsRNAs having blunt ends, 5′ overhangs, and 3′ overhangs, as well as partial dsRNAs, are equally well modified (Bass and Weintraub, 1988; Wagner *et al.*, 1989). In addition, the activity also does not distinguish between capped and non-capped RNAs (Bass and Weintraub, 1987; Wagner and Nishikura, 1988).

In this study, the substrate specificity of the dsRNA unwinding/modifying activity has been further investigated. Previous characterizations of substrate specificity have been limited to intermolecular dsRNA of relatively long lengths (Bass and Weintraub, 1988; Wagner *et al.*, 1989). While such structures form *in vivo* between sense and antisense transcripts, the more frequent occurrence of RNA duplexes is as intramolecular dsRNAs, participating in RNA secondary structures. We investigate whether such intramolecular dsRNA segments can serve as substrates for this unwinding/modifying activity and whether their length is important. We find that artificially constructed intramolecular duplexes with varying lengths of double-stranded regions are modified *in vitro*. With identical sequences and lengths of double-stranded regions, intramolecular dsRNA was found to be slightly less efficiently modified than its counterpart intermolecular dsRNA. Our results demonstrate for the first time that intramolecular dsRNA can serve as a substrate for the unwinding/modifying activity. We also found that the efficiency of adenosine modification abruptly decreased when the length of the double-stranded region of either intermolecular or intramolecular dsRNAs became shorter than 100 base pairs (bp). Short dsRNAs of 38, 23 and 15 bp were found to be modified, but very inefficiently. Thus, a relatively long double helical structure of at least 100 bp seems to be required for efficient modification, whereas the absolute minimum length of the double-stranded region for substrate recognition may be as short as 15 bp.

Results

Intramolecular dsRNA as substrate for the unwinding/modifying activity

The substrate specificity of the dsRNA unwinding/modifying activity was studied using an *in vitro* assay system (Bass and Weintraub, 1987; Wagner and Nishikura, 1988) and artificially synthesized RNA substrates. First, we investigated whether double-stranded regions of intramolecular RNAs are modified at all by the activity, since previous studies were conducted only with intermolecular dsRNAs (Bass and Weintraub, 1987, 1988; Rebagliati and Melton, 1987; Wagner and Nishikura, 1988; Wagner *et al.*, 1989, 1990).

To synthesize RNAs with defined intramolecular double-stranded regions, a full-length human α2-globin cDNA was subcloned into an *in vitro* transcription vector. Defined segments of this cDNA were then added as an insert within the 3′ non-translated region in the antisense orientation, as shown schematically in Figure 1A. We first tested three intramolecular dsRNAs containing relatively long double-stranded regions, namely 285 bp (molecule 285R), 118 bp (molecule 118R) and 70 bp (molecule 70R). Although these transcripts would be predicted to form extensive and stable secondary structures, we were concerned with the possibility that intermolecular dsRNAs might also form among multiple copies of the molecules. To minimize this possibility, all of the intramolecular dsRNAs were purified from a denaturing gel (Figure 2A), heated and ice-chilled in low salt buffer prior to analysis. In addition, we tested the efficiency of intramolecular stem formation by digesting each of these renatured transcripts with RNase A and T1 immediately after heating and ice-chilling. While the self-annealing of an intramolecular double strand occurs as a zero-order reaction, an intermolecular double strand is formed in a second-order reaction, depending on the square of the RNA concentration. We reasoned that a subpopulation of intermolecular dsRNA, if any, would therefore be decreased upon dilution of the RNA, which would be reflected in a decrease of RNase resistant double strand detected after digestion with ribonucleases and subsequent gel electrophoresis. We found no diminution in RNase resistant dsRNA despite a 100-fold dilution of the transcripts prior to reannealing. One example of such an experiment for the molecule 118R is shown in Figure 3. Thus, the level of RNase resistant dsRNAs did not depend on the RNA concentration over the range of concentrations used for the unwinding/modification assay (Figure 3), indicating that the contribution of intermolecular RNA−RNA duplex to the total dsRNA in the preparation is negligible under our conditions.

Intermolecular dsRNAs of 285 bp (molecule 285T), 118 bp (molecule 118T) and 70 bp (molecule 70T) were generated by single strand specific RNase A and T1 digestion of the appropriate transcripts and subsequent fractionation on a denaturing gel (Figures 1A and 2B). As a single-stranded RNA control, a full-length α2-globin RNA (molecule αFL) was also prepared from a parental construct. The sizes of all RNAs were confirmed on denaturing polyacrylamide gels (Figure 2A), and the sizes of the double-stranded stem portions of intramolecular dsRNAs were confirmed by their resistance to RNase A digestion (Figure 2B).

These RNAs were subjected to the *in vitro* unwinding/modification reaction using HeLa cell extracts (Manley *et al.*, 1980). In order to verify that the modification occurred on intramolecular dsRNAs, and not on intermolecular dsRNAs which might have been generated during incubation by ribonucleases in the extract, the RNAs were gel purified and analyzed after the modification reaction. We found that the intensity and sizes of all RNAs modified remained the same as those of unmodified control RNAs, suggesting that no endogenous ribonucleases are present in the HeLa extract used (data not shown). We found that both the intermolecular dsRNA (molecules 285T, 118T and 70T in Figure 1A) and the intramolecular dsRNA (molecules 285R, 118R and 70R in Figure 1A) were modified during the reaction (Figure 4). As expected, a single-stranded RNA (molecule αFL) was not modified (Figure 4, lane a). This result demonstrates for the first time that not only intermolecular but also intramolecular dsRNAs can serve as substrates for the dsRNA unwinding/modifying activity. Approximately 30% (molecule 70T) to 40% (molecule 285T) of adenosine residues were converted to inosine in the intermolecular dsRNAs. Although the level of conversion of A to I in intramolecular dsRNA (3−10%) was less than that of intermolecular dsRNA, this difference can be partly attributed to the significant proportion of the RNA which is not involved in the duplex and hence not subject to

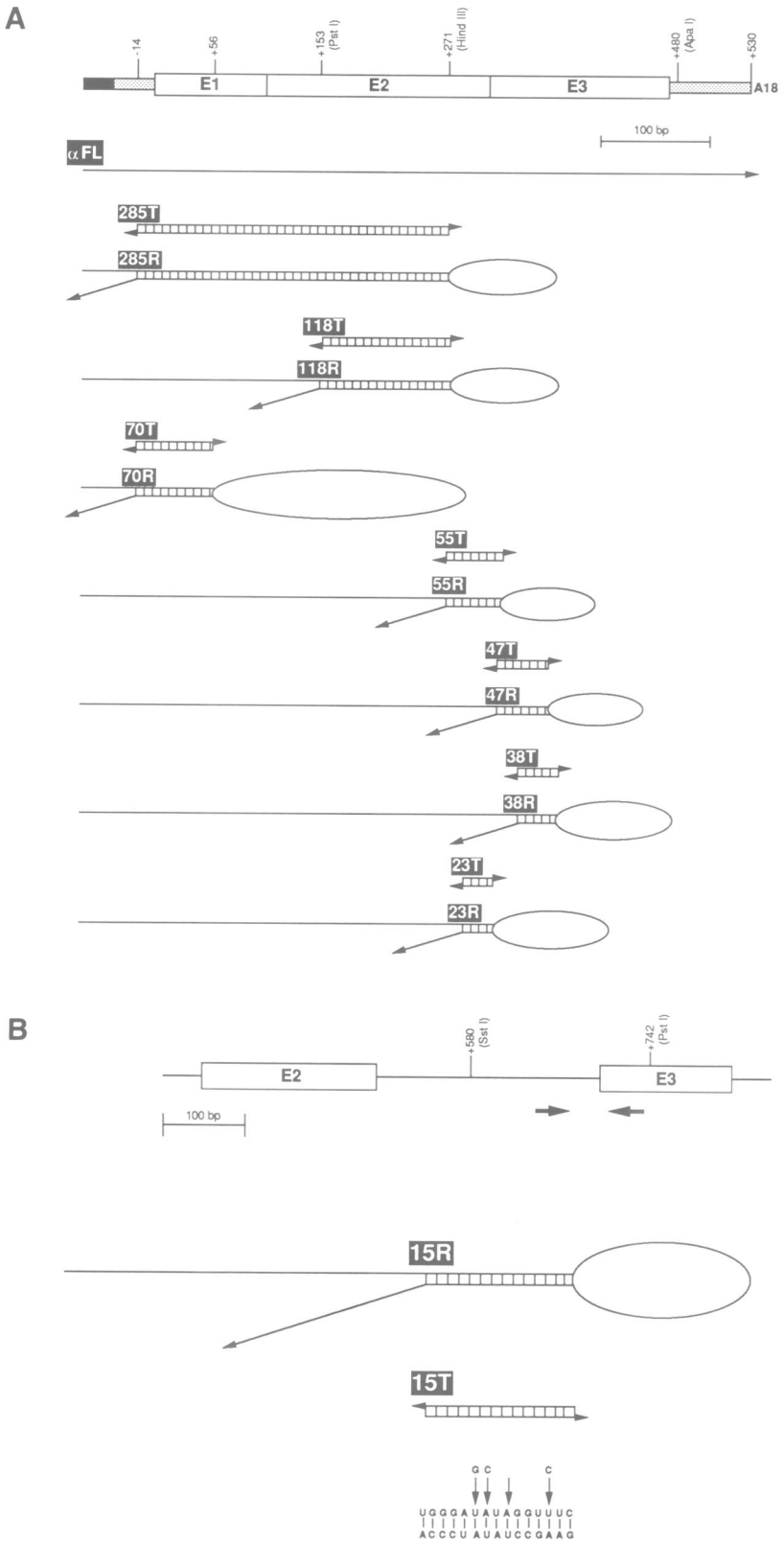

Fig. 1. Schematic presentation of synthetic α2-globin and human growth hormone RNAs with intermolecular or intramolecular double-stranded regions. Sets of intermolecular (T series molecules) and intramolecular (R series molecules) with identical sequences and length of double-stranded regions were prepared from plasmid constructs carrying α2-globin or growth hormone gene sequence. (**A**) α2-globin RNAs. A cDNA encoding the human full-length α2-globin cDNA or its derivatives were used as templates for the synthesis of the α2-globin RNA (αFL) or its derivatives with intramolecular double-stranded regions of 285, 118, 70, 55, 47, 38 and 23 bp (285R, 118R, 70R, 55R, 47R, 38R and 23R) as described in Materials and methods. All intermolecular dsRNAs were generated by digesting single-stranded portions of corresponding intramolecular dsRNAs and subsequent purification of RNase resistant dsRNAs from a denaturing polyacrylamide gel. (**B**) Human growth hormone RNAs. The shortest RNAs, containing 15 bp of intramolecular (15R) and intermolecular (15T) double-stranded regions, were made from a construct carrying the mutagenized human growth hormone gene as described in Materials and methods.

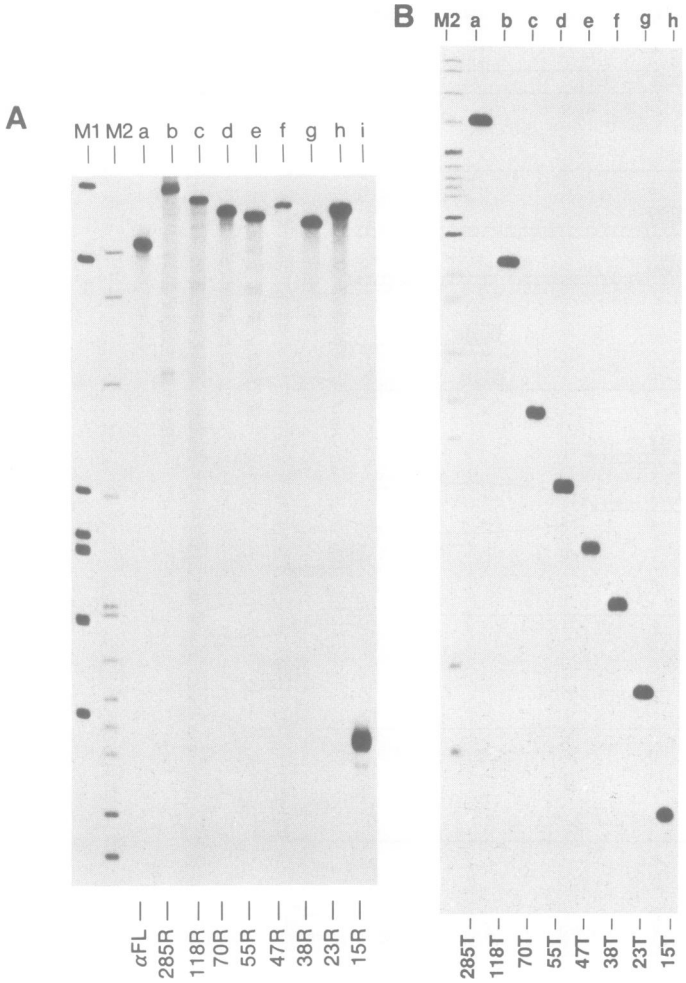

Fig. 2. Preparation of a series of intermolecular and intramolecular dsRNAs. Capped RNAs labeled with [α-^{32}P]ATP were prepared by *in vitro* transcription of linearized α2-globin and human growth hormone gene constructs and subsequent purification on a preparative denaturing polyacrylamide gel. Blunt-ended intermolecular dsRNAs were prepared by digesting corresponding intramolecular dsRNAs with RNase A and T1 and subsequently isolating the RNase resistant RNAs from a preparative denaturing polyacrylamide gel. The RNAs were then annealed together to form intermolecular dsRNAs (see Materials and methods). (**A**) All intramolecular dsRNAs used in this study were fractionated on a 4% polyacrylamide denaturing gel. Total sizes of these RNAs were 620 (αFL), 905 (285R), 738 (118R), 690 (70R), 675 (55R), 714 (47R), 658 (38R), 689 (23R) and 178 nt (15R). Note that 47R and 23R contain two and three tandem repeat inserts in the antisense orientation. In addition, because of the strong tendency to form a secondary structure, some RNAs generated a second band in addition to the expected size RNA (denatured) band despite the presence of 8.3 M urea and with the high temperature (50°C) used for electrophoresis. For instance, see lane b for molecule 285R. (**B**) All intermolecular dsRNAs prepared were analyzed on a 10% polyacrylamide denaturing gel. Lanes M1 and M2, size markers: 5′-^{32}P-labeled ϕX174 *Hae*III digests and pBR322 *Msp*I digests, respectively.

modification (Bass and Weintraub, 1988; Wagner *et al.*, 1989). When this factor is taken into account (see Materials and methods), the modification efficiencies of three intramolecular dsRNAs were estimated as 17, 17 and 15% for 285R, 118R and 70R molecules, respectively.

Relative efficiency for modification of intermolecular and intramolecular dsRNAs

When we compared the efficiency of the modification of a set of intermolecular and intramolecular dsRNAs containing identical double-stranded regions, the modification of the intramolecular dsRNA appeared to be less efficient than that of the counterpart intermolecular dsRNA; e.g. 30% A to I conversion for 70T versus 15% for 70R. This difference could be due to the different affinity of the unwinding/modifying activity for intermolecular and intramolecular dsRNAs. Alternatively, the result may reflect the inability of the activity to modify adenosine residues in certain regions of intramolecular dsRNA. For instance, while both sense and antisense strands of intermolecular dsRNAs were shown previously to be equally modified (Bass and Weintraub, 1988; Wagner *et al.*, 1989), only one half of the double-stranded region of intramolecular dsRNA might be modified. To address these issues, we first analyzed the modification efficiency of a set of intermolecular and intramolecular dsRNAs (70T and 70R) with varying concentrations of enzyme. We found that the double-stranded regions of intramolecular dsRNA can be modified to an extent similar to that of intermolecular dsRNA if more enzyme is used (Figure 5). The relative efficiency of modification of intermolecular and intramolecular dsRNAs was also analyzed by a competition assay. It has been shown previously that the reaction of a radioactively labeled intermolecular dsRNA is inhibited by adding an excess of a second unlabeled intermolecular dsRNA, whereas it is not inhibited by the addition of a similar amount of single-stranded RNA, single- or

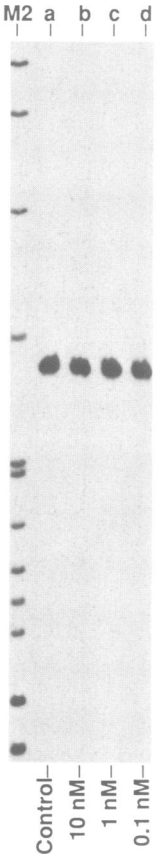

Fig. 3. Intramolecular double strand formation is dominant over intermolecular double strand. An intramolecular dsRNA, 118R, was used to confirm that the RNA indeed forms predominantly intramolecular double strands rather than intermolecular double strands. The RNA was heated at 80°C in double distilled water and then diluted into ice-chilled unwinding/modifying reaction buffer (see Figure 4 legend) to make 0.1–10 nM RNA final concentration. Immediately, RNase A (40 μg/ml) and RNase T1 (2 μg/ml) were added to each tube and the RNAs were digested at 30°C for 30 min. The RNase resistance products (118 bp dsRNAs) were fractionated on a 4% polyacrylamide denaturing gel. Note that the intensity of RNA bands derived from analysis carried out at three different RNA concentrations is very similar to that of the control, in which an equal amount of the RNA was digested with RNases in a similar way except that heating and ice-chilling steps were excluded.

double-stranded DNA, or tRNA (Bass and Weintraub, 1987; Wagner and Nishikura, 1988). In this experiment, various concentrations of the cold 70T intermolecular dsRNA were preincubated with a fixed amount of enzyme prior to the addition of [^{32}P]ATP-labeled 70T intermolecular or 70R intramolecular dsRNA. As shown in Figure 6, the relative concentration of the intermolecular dsRNA (70T) competitor required for abolition of modification of 70R was lower than for 70T, suggesting that the binding affinity of the dsRNA unwinding/modifying activity for the intermolecular dsRNA is higher than for a counterpart intramolecular dsRNA carrying a double-stranded region of identical sequence and length. Taken together, our results from these two experiments suggest that the relative efficiency of modification of intramolecular dsRNA is slightly less than that of intermolecular dsRNA because of lower binding affinity of the enzyme to intramolecular than intermolecular dsRNA. However, with higher concentrations of the enzyme, the intramolecular dsRNA can be modified to a similar extent as the intermolecular dsRNA.

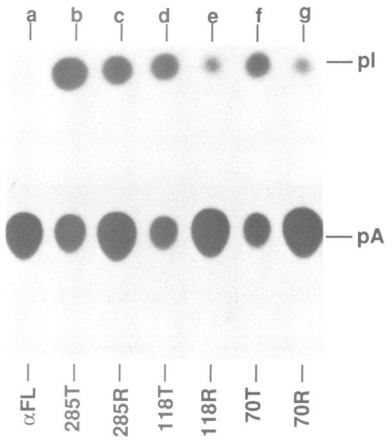

Fig. 4. Modification of both intermolecular and intramolecular dsRNAs. The unwinding/modifying assay was performed at 37°C for 2 h using conditions described previously (Wagner *et al.*, 1989). The reaction mixture contained 5 fmol of [α-^{32}P]ATP-labeled RNA and 50 μg of HeLa cell extract prepared as described previously (Manley *et al.*, 1980). The unwound/modified RNAs were digested to 5′-mononucleotides with P1 nuclease, and the reaction products were next chromatographed on TLC plates as described in Materials and methods. The radioactivity of each adenosine (pA) and inosine spot (pI) was quantified by a radioanalytical imaging system (AMBIS, San Diego, CA). The estimated inosine conversion was 40, 40 and 30% for 285T, 118T and 70T intermolecular dsRNA, respectively. Intramolecular dsRNAs, 285R, 118R and 70R, contain some adenosine residues not engaged in base pairing (65 of 162 A residues in 285R, 98 of 134 A residues in 118R, and 104 of 131 A residues in 70R). When only adenosine residues in the double-stranded region were considered, the inosine conversions were estimated to be 17, 17 and 15% for 285R, 118R and 70R, respectively.

Length requirement for substrate dsRNA

In addition to the difference of modification efficiency between intermolecular and intramolecular dsRNAs, we also noticed a decreased modification efficiency of 70T and 70R compared with longer substrates, namely 285T and 285R or 118T and 118R (Figure 4), suggesting that there may be a requirement for a certain length of double-stranded regions in the substrate RNA. In our previous studies, two different intermolecular dsRNAs, a 575 bp segment of mouse c-*myc* dsRNA and a 339 bp segment of rabbit β-globin dsRNA, were used and found to be modified equally well, ~50% A to I conversion (Wagner *et al.*, 1989). The present experiments suggest, however, that RNA molecules with double-stranded regions shorter than 118 bp may be less efficient substrates. In order to test the possible correlation of the size of double-stranded regions with modification efficiency, we prepared four additional sets of intermolecular and intramolecular α2-globin dsRNAs containing double-stranded regions shorter than 70 bp (Figure 1A): 55, 47, 38 and 23 bp. In addition, we synthesized another set of dsRNAs containing a very short (15 bp) double-stranded region by transcription of a construct carrying an *in vitro* mutagenized segment of the human growth hormone gene (15R), and by RNase A and T1 digestion of the transcript (15T) (Figure 1B). These shorter dsRNAs together with the longer dsRNAs used earlier were subjected to the *in vitro* unwinding/modifying assay. For these experiments, we used extensively purified dsRNA unwinding/modifying activity prepared from bovine liver nuclear extracts (at least 20 000-fold enriched; U.Kim and K.Nishikura, manuscript in preparation) instead of HeLa extracts in order

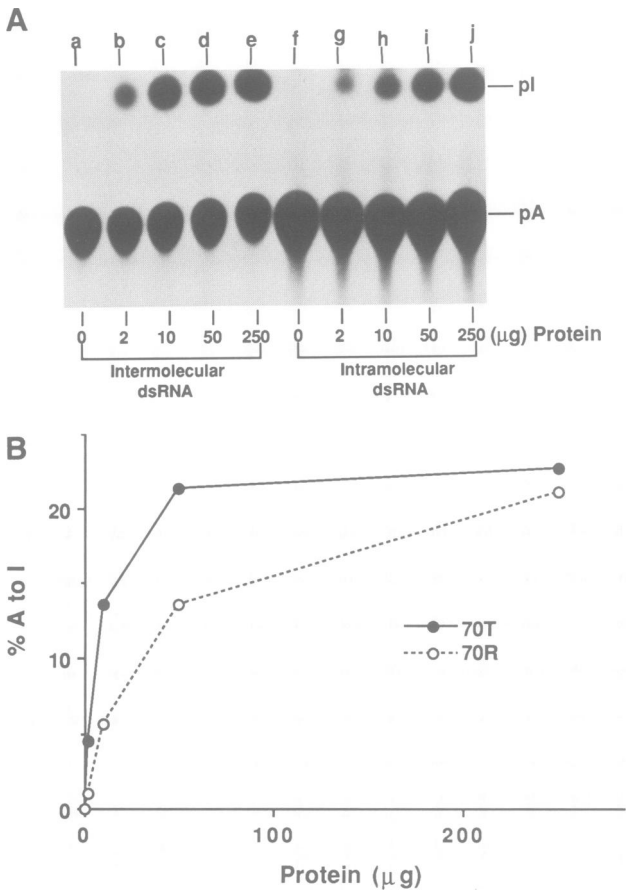

Fig. 5. Effect of protein concentration on modification of intermolecular and intramolecular dsRNAs. A set of intermolecular and intramolecular dsRNAs (70T and 70R) was tested for modification with various amounts of protein (HeLa cell extract). (**A**) The unwinding/modifying assay was carried out as described in Figure 4. (**B**) The amounts of A to I conversion were plotted against extract protein concentration. Although the initial velocity for modification of intramolecular dsRNA appears to be slightly lower than that for intermolecular dsRNA under these conditions, a similar extent of modification was obtained with the highest protein concentration (250 μg) tested.

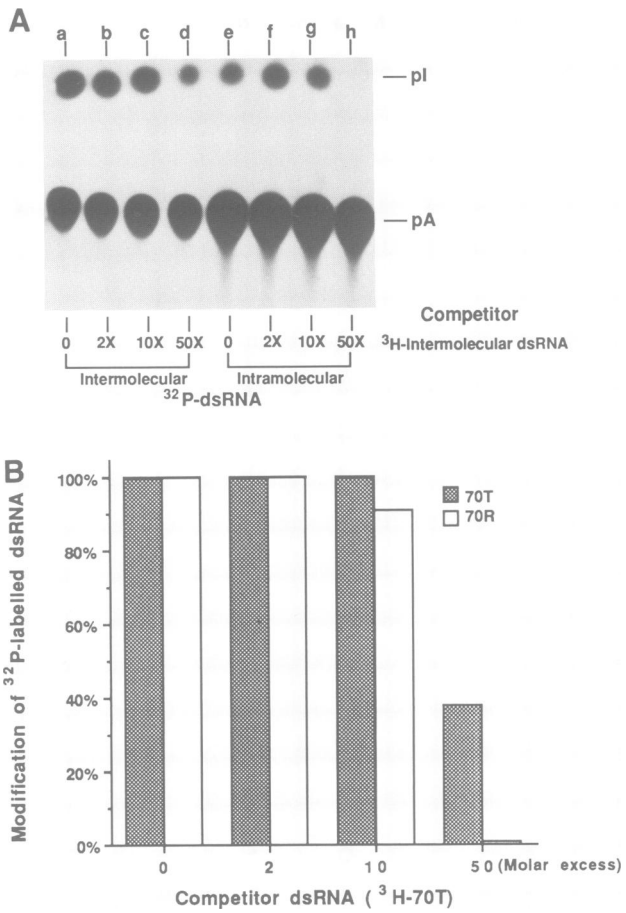

Fig. 6. Competition between intermolecular and intramolecular dsRNAs. A set of intermolecular and intramolecular dsRNAs (70T and 70R) was tested for modification competition. (**A**) The reaction mixture containing 10 μg of HeLa cell extract protein was preincubated with various amounts of [^{3}H]UTP-labeled 70T at 37°C for 15 min, and then 5 fmol of [^{32}P]ATP-labeled 70T or 70R was added for an additional 1 h incubation. (**B**) The efficiency of modification of [^{32}P]ATP-labeled 70T or 70R relative to its control (no competitor) was estimated and plotted against various concentrations of [^{3}H]UTP-labeled competitor 70T dsRNA. With a 50-fold molar excess of competitor 70T dsRNA, modification of 70R intramolecular dsRNA was completely inhibited, whereas modification of 70T intermolecular dsRNA, though decreased, still remained detectable.

to keep the enzyme concentration relatively high. With the assay conditions used, we confirmed that two longer intermolecular dsRNAs, 285T and 118T, could be modified to nearly their full extent, i.e. ~45%, whereas intermediate size intermolecular dsRNAs, 70T, 55T and 47T molecules underwent A to I modifications of ~40, 35 and 10%, respectively. Shorter dsRNAs, 38T, 23T and 15T, were very inefficiently, or not at all, modified, resulting in 5, 1 and 0% of adenosine residues converted to inosine (Figure 7A). We found a similar size dependence of the modification efficiency for the counterpart intramolecular dsRNAs (Figure 7B). With the assay conditions used, the maximum modification obtained for 118R and 285R was still less than that of their intermolecular counterparts. On the other hand, short intramolecular dsRNAs (38R and 23R) were modified slightly more than their counterpart intermolecular dsRNAs (38T and 23T). When the short intramolecular dsRNAs, 23R and 15R, and the short intermolecular dsRNA, 23T, were analyzed once again but with 3-fold higher enzyme and 10-fold higher substrate concentrations, they were confirmed to be modified to a small extent, whereas intermolecular dsRNA 15T was not detectably modified (Figure 7C).

We carried out two additional experiments to exclude the possibility that the short dsRNAs may not remain base paired during the unwinding/modifying reaction, which would result in their inability to be modified. First, all short dsRNAs found to be inefficient substrate RNAs were treated with RNase A and T1 at the end of the unwinding/modification reaction in the same reaction tube, and then the reaction products were analyzed by electrophoresis on a 20% polyacrylamide gel. All of the double-stranded regions of these RNAs were found to remain RNase resistant, suggesting that they remained double-stranded during the reaction (data not shown). Second, the short intermolecular dsRNAs were examined on a native 10% polyacrylamide gel immediately after the unwinding/modifying reaction. As seen from an example of such analysis for the 23T molecule (Figure 8), we found that this short intermolecular dsRNA remains double-stranded during the reaction. From these experiments, we concluded that there is a size requirement for efficient modification of substrate RNAs, both inter-

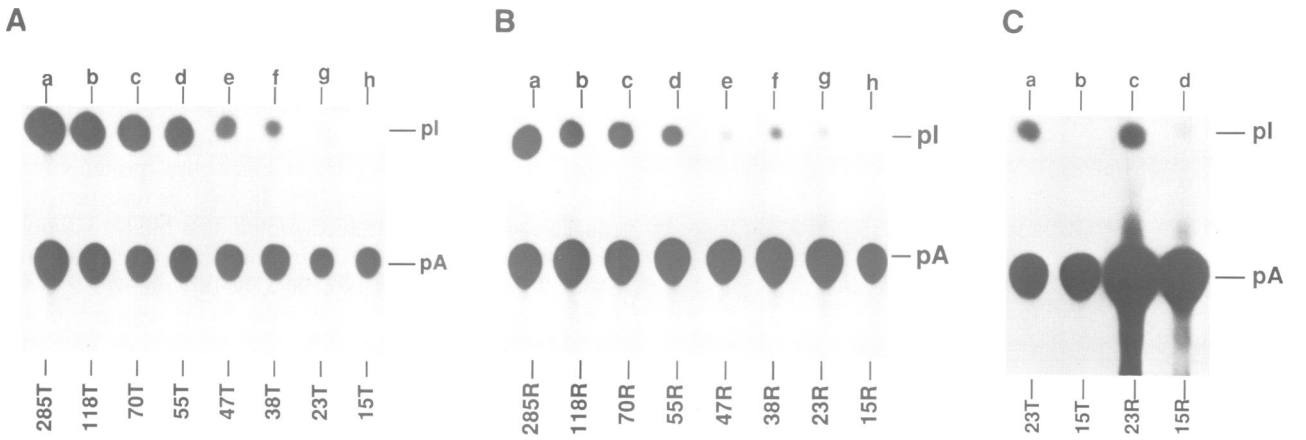

Fig. 7. Length requirement of substrate RNA for the unwinding/modifying activity. A series of substrate RNAs containing varying sizes, 285 bp to 15 bp, of either intermolecular (**A**) or intramolecular (**B**) double-stranded regions were examined for the relation between modification efficiency and lengths. The unwinding/modifying assay was carried out as described in Figure 4, except that 10 ng of partially purified enzyme (20 000-fold enriched) from bovine liver nuclear extracts (U.Kim and K.Nishikura, manuscript in preparation), which is approximately equivalent to 150 μg of HeLa whole cell extract proteins, was used as enzyme source. The percent inosine conversion estimated for modification of intermolecular dsRNAs was ~45, 40, 40, 35, 10, 5, 1 and 0% for 285T, 118T, 70T, 55T, 47T, 38T, 23T and 15T, respectively. The percent inosine conversion estimated for modification of intramolecular dsRNAs was ~25, 25, 25, 17, 7, 7, 3 and 1% for double-stranded regions of 285R, 118R, 70R, 55R, 47R, 38R, 23R and 15R, respectively. (**C**) The two shortest intermolecular (23T and 15T) and intramolecular (23R and 15R) dsRNAs were analyzed once again but with a higher concentration of substrate RNAs (50 fmol instead of 5 fmol) and a higher enzyme concentration (30 ng of partially purified unwinding/modifying activity).

molecular and intramolecular dsRNAs. The size limit for efficient modification became more clear when the results of base modification analysis of all dsRNAs were plotted against the size of double-stranded regions (Figure 9). The efficiency of modification of dsRNAs decreases abruptly when the double-stranded region becomes < 100 bp. Substrate dsRNAs of 38 bp or shorter are modified inefficiently. It is likely that this observed lower limit of substrate dsRNA length may depend on individual sequence context. For instance, short dsRNA with particularly favorable sequences such as AU-rich regions (Kimelman and Kirschner, 1989), may serve as a better substrate than the dsRNAs used in this study, which contain essentially random sequences. Furthermore, as seen for modification of some of the longer substrates, the modification efficiency may depend on the assay conditions such as enzyme or substrate concentrations, and it is still possible that dsRNA of 15 bp or even less could be modified under some as yet untested conditions. Nevertheless, our results suggest that the efficiency of modification of both intramolecular and intermolecular dsRNA is critically dependent on substrate length and that there may be an absolute minimum size required for substrate recognition.

Discussion

In this study, we have shown by *in vitro* assay that the dsRNA unwinding/modifying activity can modify not only intermolecular but also intramolecular dsRNA. This has important implications concerning the natural substrate RNAs for this activity. Our results have dramatically widened the knowledge of the possible range of substrate RNAs, which now include hairpin type RNAs with intramolecular double-stranded regions in addition to intermolecular double strands made from complementary sense and antisense strand RNAs. In addition, we found that the efficiency of modification abruptly drops for substrate RNAs containing double-stranded regions shorter than ~100 bp. An intermolecular dsRNA of 15 bp was found

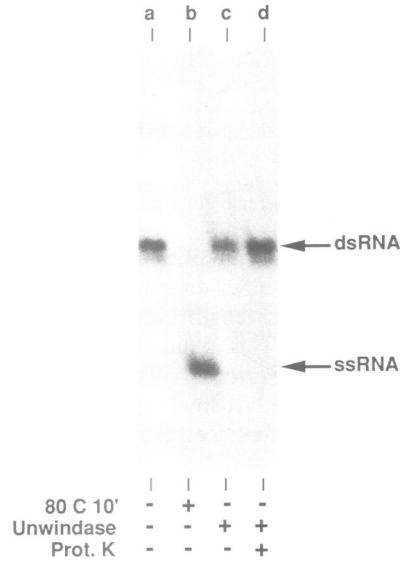

Fig. 8. Maintenance of double-strandedness during the unwinding/modifying reactions of a short intermolecular dsRNA. The short intermolecular dsRNA of 23 bp (molecule 23T), 10 fmol, was subjected to the unwinding/modifying reaction using partially purified enzyme (U.Kim and K.Nishikura, manuscript in preparation), as described in the legend to Figure 4. One half of the reaction products was further incubated with 1 mg/ml of proteinase K for 30 min at 37°C (lane d). A portion of the reaction products (2 fmol) was loaded directly onto a native 10% polyacrylamide gel (Wagner and Nishikura, 1988) and the RNAs were fractionated by electrophoresis. As controls, an equivalent amount of 23T RNA in the reaction buffer but without enzyme was examined without (lane a) or with heating at 80°C for 10 min prior to loading. Note that the majority of 23T RNA remains double-stranded instead of being dissociated into single strands (lane d). In lane c, some of the dsRNAs appeared to be bound with protein(s) due to the omission of proteinase K treatment, which resulted in slow-migrating smears and a decrease in the major dsRNA band intensity.

not to be modified at all using a variety of enzyme and substrate concentrations, while short intermolecular and intramolecular dsRNAs of 23 bp, and a short intramolecular

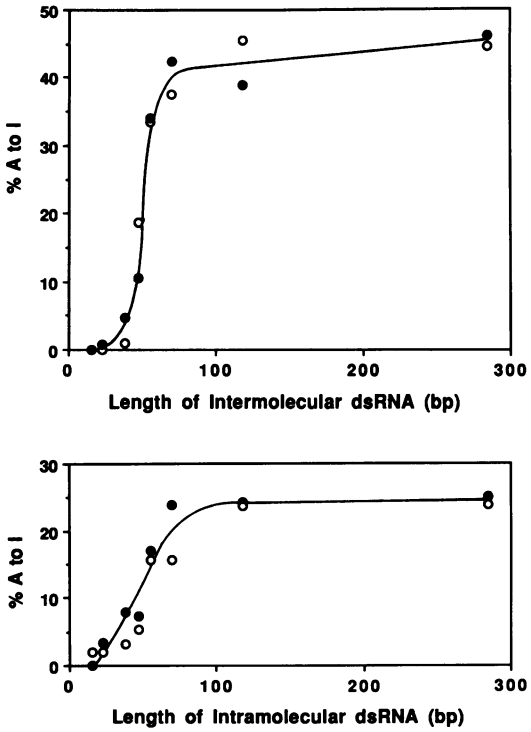

Fig. 9. Modification efficiency for various sizes of intermolecular and intramolecular dsRNAs. The modification efficiency of each intermolecular or intramolecular dsRNA shown in Figure 7A or B was estimated as described in the legend to Figure 4. (Note that the efficiencies for intramolecular dsRNAs were calculated only on the basis of adenosines within the double-stranded regions of these molecules.) The results from Figure 7A (filled circle) and from a second set of similar experiments (open circle) were plotted against the size of the double-stranded region of each molecule.

dsRNA of 15 bp, were modified but very inefficiently. It seems that the dsRNA unwinding/modifying activity requires an absolute minimum length between 23 and 15 bp for substrate recognition. This minimal size constraint is similar to that seen for *Escherichia coli* RNase III, one of the best characterized dsRNA binding enzymes. This enzyme requires 20−25 bp of perfect double-stranded region as a substrate (Robertson, 1982). This minimum target size includes two turns of the dsRNA helix (Robertson, 1982).

There are two striking features of the change in dsRNA modification efficiency with length of double-stranded region as graphed in Figure 9. First, the plateau of maximal modification does not occur until the double-stranded region is surprisingly long, >100 bp. This represents 10−12 turns of the dsRNA double helix, i.e. a target of ~30 nm in length. Second, the decline in modification efficiency below 100 bp is extraordinarily abrupt. This transition is much too steep to be accounted for by any simple 'end-effect' (e.g. modification at reduced or zero efficiency within some fixed distance of the ends of each substrate molecule) and the control experiments described above seem to rule out artifacts such as incomplete hybridization of the short double-stranded regions. This highly non-linear dependence of modification efficiency on target size suggests some co-operative interaction extending over a long distance, of the order of 50−100 bp. The large distances and extreme co-operativity implied by the data shown in Figure 9 suggest that more than a single enzyme molecule may be involved in the active complex.

Preliminary evidence from our ongoing efforts to purify the unwinding/modifying activity suggests a molecular weight in the range of 100 kDa (U.Kim and K.Nishikura, manuscript in preparation). It would be highly unusual for an enzymatic protein of this molecular weight to have a length as great as 30 nm, though even larger length/mass ratios are known among fibrous structural proteins (e.g. tropomyosin: 38 nm, 70 kDa). It may be instructive to consider another well studied protein that binds to double-stranded nucleic acid in a co-operative manner, such as the SV40 T antigen. This is a 96 kDa protein which binds strongly as a tetramer to a defined 26 bp sequence of the SV40 DNA near its origin of replication and early transcriptional promoter. Strong co-operativity in binding results from interaction between three tetrameric complexes binding to three of the 26 bp sequences, which are separated from each other by ~20 bp along the DNA (Tjian, 1981; Myers *et al.*, 1981). A similar interaction among supramolecular complexes of the dsRNA unwindase could account for the large apparent target size and extreme sensitivity to changes in length of the double-stranded region that we show in Figure 9. It may be possible to collect further evidence on this point by examining the rate of dsRNA modifications at a series of very low enzyme concentrations. If extensive co-operative interactions occur between different dsRNA unwindase molecules on a single substrate molecule, then one would expect a highly non-linear dependence of reaction rate on enzyme concentration and on enzyme/substrate ratio.

Since naturally occurring intramolecular dsRNAs present in viral RNAs or eukaryotic mRNAs usually contain relatively short and often imperfect double-stranded regions, it is questionable whether they serve as substrates for the unwinding/modifying activity. To address this issue, we have recently examined *in vitro* synthesized short RNAs containing HIV-1 TAR and RRE (Cullen and Greene, 1989; Sharp and Marciniak, 1989). The TAR sequence, located between +1 and +59 of all HIV-1 mRNAs (Muesing *et al.*, 1987; Feng and Holland, 1988), forms a stable stem−loop structure, which is required for *tat* transactivation (Peterlin *et al.*, 1986; Muesing *et al.*, 1987; Cullen and Greene, 1989; Sharp and Marciniak, 1989). The RRE sequence, located within the coding region of the *env* gene (Rosen *et al.*, 1988), is predicted to have a complicated secondary structure with a central stem and five additional stem−loops (Dayton *et al.*, 1989; Malim *et al.*, 1989), which is hypothesized to regulate RNA transport or splicing by interacting with *rev* regulatory protein (Sodroski *et al.*, 1986; Rosen *et al.*, 1988; Chang and Sharp, 1989; Dingwall *et al.*, 1989; Emerman *et al.*, 1989; Felber *et al.*, 1989; Malim *et al.*, 1989). Initially we could not observe any adenosine modification in these two HIV-1 RNA elements with standard assay conditions, as described in Figure 7. With ~20-fold higher substrate and 3-fold higher enzyme concentrations, however, a very small fraction (<1%) of adenosine residues of these viral RNAs was found to be modified (K.Nishikura, C.Dingwall and A.Dayton, unpublished results). It is currently not known whether the limited modification of these two viral RNA secondary structures observed *in vitro* has any significance *in vivo*. One possibility that must be kept in mind is that the naturally occurring hairpins in these RNAs may be stabilized or in some way altered *in vivo* to form more helix-like structures, and thus allow the dsRNA unwindase to recognize them as substrate RNAs. This would parallel the case of bacteriophage T7 rRNA precursor which serves as a natural substrate

RNA for RNase III (Dunn and Studier, 1981; Robertson, 1982). RNase III normally requires its substrate to have two complete turns of dsRNA double helix. Surprisingly, one of the RNase III recognition sites found in phage T7 mRNA precursor does not contain a clear two-turn dsRNA helix structure, but the RNA mimics a dsRNA structure *in vivo* by incorporating non-Watson–Crick structural features and by a folding back of each strand in the nine unpaired regions (Dunn and Studier, 1981; Robertson, 1982). A similar adjustment of the RNA structure could take place *in vivo* with substrate dsRNAs for the unwinding/modifying activity, possibly in interaction with other cellular factors that recognize and bind to specific dsRNA structures. In this connection, it is interesting to note that an adenosine residue of HIV-1 TAR RNA is modified to inosine when the RNA is co-injected with *tat* proteins into *Xenopus laevis* oocyte nuclei (L.Sharmeen and M.Groudine, personal communication). Thus, in future experiments it will be important also to examine the substrate specificity of the dsRNA unwinding/modifying activity *in vivo*, which may be different from that tested *in vitro*.

Materials and methods

Plasmid construction

A cDNA encoding the full-length α2-globin cDNA constructed in the SP64 vector (SP64αFL) was used to prepare the derivative cDNAs containing duplicated segments, which were used as templates for the synthesis of various α2-globin RNAs with intramolecular double-stranded regions. The assembly of this full-length α2-globin cDNA and the derivative cDNAs containing duplicated segments is described in detail elsewhere (S.Liebhaber, F.Cash and S.Eschelman, manuscript in preparation). Briefly, coding region segments of the α2-globin cDNA were isolated by restriction endonuclease digestion, ligated to *Apa*I linker and inserted at a unique *Apa*I site (+480) in the 3′ non-translated region in the antisense orientation. The duplicated fragment in molecule 285R extends from −14 to +271; in molecule 118R from +153 to +271; in molecule 70R from −14 to +56; in molecule 55R from +271 to +325; in molecule 47R from +321 to +367; in molecule 38R from +330 to +367; and in molecule 23R from +291 to +313. The termini of each of the inserted fragments were confirmed by sequence analysis and the orientation of the insert within the *Apa*I site relative to the α2-globin cDNA (sense or antisense) was determined by restriction mapping. In molecules 47R and 23R, it was found that two and three tandem repeats of insert DNA fragments were created during the ligation reaction, respectively, while only a single copy of the fragment was inserted in all other molecules.

pGEM(N-ds) carrying a short inverted repeat, artificially introduced into a fragment of human growth hormone gene, was prepared as follows. A short genomic DNA fragment (*Sst*I–*Sma*I, 294 bp), excised from the human growth hormone gene, hGH-N (Estes *et al.*, 1990), was used as a template for site-directed mutagenesis (Zoller and Smith, 1983). This segment extends from intron 2 to exon 3 and into intron 3. To create a perfect inverted repeat of 15 bp (as shown by the two thick arrows in the upper diagram of Figure 1B), three nucleotide substitutions and a single base insertion were introduced into this segment (as shown in the lower diagram of Figure 1B). The mutagenized DNA fragment was ligated into a SP6 vector (pGEM3) and digested with *Sst*I and *Sma*I, resulting in pGEM(N-ds).

Preparation of intermolecular and intramolecular dsRNAs

All α2-globin construct plasmids (SP64αFL and its derivatives) were linearized at a unique *Sal*I site located near the 3′ end of the cDNA insert. The human growth hormone construct, pGEM(N-ds), was linearized with *Pst*I within the third exon of hGH-N, and the end was filled in with DNA polymerase Klenow fragment in order to avoid non-specific end-to-end transcription. Capped RNAs labeled with [α-^{32}P]ATP or [^{3}H]UTP were transcribed from each of the plasmids *in vitro*, as described previously (Wagner and Nishikura, 1988), resulting in either a full-length α2-globin RNA (single-stranded) or a series of RNA molecules containing varying sizes of intramolecular double-stranded regions. All intramolecular dsRNAs, as well as the full-length α2-globin RNAs, were purified after *in vitro* transcription by electrophoresis on a 6% polyacrylamide denaturing gel, and dissolved in double distilled water followed by heating to 80°C for 10 min and then ice-chilled in order to avoid any intermolecular dsRNA formation.

In vitro transcription, using pGEM(N-ds) as template DNA, generated an RNA containing an intramolecular perfectly base paired short stem (15 bp), single-stranded overhangs of 85 and 26 nucleotides and a 37 nucleotide loop. The transcript (178 nucleotides in length) was also purified on a denaturing 6% polyacrylamide gel.

All intermolecular dsRNAs were prepared as follows. Intramolecular dsRNAs were first digested with RNase A (40 μg/ml) and T1 (2 μg/ml) at 15°C for 2 h, and then the RNase resistant blunt-ended stems were isolated from a 10% denaturing polyacrylamide gel. The RNAs were dissolved in buffer containing 20 mM Tris, pH 7.5, and 0.15 M NaCl, heated at 80°C for 10 min, and cooled slowly to room temperature to allow the annealing of sense and antisense strands. The double-strandedness of the intermolecular dsRNAs thus prepared was confirmed once again by their resistance to the RNase A and T1 treatment.

Unwinding/modification assay

dsRNA unwinding/modifying activity was assayed *in vitro* (Bass and Weintraub, 1987; Wagner and Nishikura, 1988). Unless specified, the reaction was carried out in 50 μl and contained 5 fmol of [^{32}P]ATP-labeled dsRNA, 50 mM Tris, pH 7.8, 0.15 M KCl, 5 mM EDTA, 1 mM dithiothreitol and 25% glycerol, and various amounts of either HeLa whole cell extracts (Manley *et al.*, 1980) or purified unwinding/modifying activity (20 000-fold enriched from bovine liver nuclease; U.Kim and K.Nishikura, manuscript in preparation). After incubation for 2 h at 37°C, the reaction products were deproteinized and then precipitated with ethanol, as described previously (Wagner and Nishikura, 1988; Wagner *et al.*, 1989).

Competition assay

The relative binding affinity of intermolecular and intramolecular dsRNA was examined by a competition assay as described previously (Bass and Weintraub, 1987; Wagner and Nishikura, 1988). Varying amounts of cold competitor intermolecular dsRNA ([^{3}H]UTP-labeled 70T) were preincubated with 10 μg of HeLa extract proteins at 37°C for 15 min prior to addition of 5 fmol of [^{32}P]ATP-labeled intermolecular (70T) or intramolecular (70R) dsRNAs. After incubation at 37°C for 1 h, the RNAs were extracted and examined by a base modification assay, and the extent of inhibition was estimated from the extent of decrease of the amount of adenosine-to-inosine conversion.

Base modification assay

After the unwinding/modifying reaction, RNA samples, together with 10 μg of *E.coli* tRNA, were digested with nuclease P1 into 5′-mononucleotides. The digests were analyzed by one-dimensional TLC. The solvent system used was 0.1 M sodium phosphate (pH 6.8)/ammonium sulfate/1-propanol, 100:60:2 (v/w/v) as described previously (SilberKlang *et al.*, 1979). The radioactivity of each adenosine and inosine spot was quantified directly by a radioanalytical imaging system (AMBIS, San Diego, CA, USA). Intramolecular dsRNAs used in this study contain some adenosine residues not engaged in base pairing; 65 of 162A in 285R, 98 in 134A in 118R, 104 of 134A in 70R, 110 of 131A in 55R, 124 of 135A in 47R, 118 of 126A in 38R, 124 of 136A in 23R and 44 of 53A in 15R. Only adenosine residues in the double-stranded region were considered for quantification of modification efficiency of intramolecular dsRNAs.

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