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A double-stranded RNA unwinding activity introduces structural alterations by means of adenosine to inosine conversions in mammalian cells and *Xenopus* eggs

(I-U base pair/"unwindase"/antisense RNA/deaminase/mismatched base-pairing)

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ABSTRACT **Amphibian eggs and embryos as well as mammalian cells have been reported to contain an activity that unwinds double-stranded RNA. We have now found that adenosine residues have been modified in the RNA products of this unwinding activity. Although the modified RNA remains double-stranded, the modification causes the RNA to be susceptible to single-strand-specific RNase and to migrate as a retarded smear on a native polyacrylamide electrophoresis gel. The modification is specific for double-stranded RNA. At least 40% of the adenosine residues can be modified *in vitro* in a given random sequence RNA molecule. By using standard two-dimensional TLC and HPLC analyses, the modified base has been identified as inosine. Mismatched base-pairing between inosine and uridine appears to be responsible for the observed characteristics of the unwound RNA. The biological significance of this modifying activity and also of the modified double-stranded RNA is discussed.**

Recently the detection of a double-stranded RNA (dsRNA) unwinding activity in mammalian cells and amphibian eggs was reported (1–3). The unwinding activity has been analyzed by an *in vitro* assay system using crude cell extracts and duplex RNA formed by sense and antisense complementary RNA strands (1, 3). After incubating the duplex RNA with the cell extract, the unwound RNA is detected both by its retarded smear on a native polyacrylamide gel and by its sensitivity to single-strand-specific RNase. The unwinding activity is abolished by proteinase treatment, suggesting that a protein is involved, and appears not to require ATP. The activity is specific for dsRNA: it is inhibited by preincubation of the reaction mixture with a 50-fold molar excess of dsRNA but not by single-stranded RNA, double-stranded DNA, single-stranded DNA, or tRNA (1, 3). The unwinding activity seems not to have sequence specificity: it unwinds different types of dsRNA formed *in vitro* from sense and antisense RNAs of β-globin, c-*myc*, and chloramphenicol acetyltransferase (1–3). The unwinding activity is low in quiescent mouse fibroblast 3T3 cells but increases when the cells are stimulated to renew growth by serum, suggesting that it may be regulated in a cell-cycle-dependent manner (3).

In our previous report, we noted several odd characteristics of the unwound RNA. The dsRNA is never completely dissociated to monomer form even by including excess protein, longer incubation times, or additional fresh protein added to previously unwound RNA for an extended length of time (3). If the unwound RNA is heated to 90°C in 90% formamide, however, it is fully denatured into full-length monomer form. In addition, the unwound RNA does not hybridize back to its native state when allowed to reanneal in the hybridization conditions used originally to form the duplex. These puzzling observations led us to infer that the unwound RNA was not completely denatured but was somehow modified to a form that was incapable of complete rehybridization (3).

In the present study these findings are extended to demonstrate that the unwound RNA is chemically modified at adenosine residues. By using two-dimensional TLC and HPLC methods, the modified base was found to be inosine. The conversion of adenosine residues to inosine residues by the unwinding activity occurs only on dsRNA. Both sense and antisense strands of the duplex are substrates for modification. The unwinding/modifying activity thus changes the base composition of the RNA, thereby introducing I-U base-pair mismatches and locally disrupting the duplex RNA structure.

MATERIALS AND METHODS

RNA Duplex Unwinding Assay and Analysis of RNA. The duplex RNA was prepared by hybridizing sense and antisense RNAs (c-*myc* and β-globin), which were synthesized *in vitro* using pSP64Mcmyc.s, pSP65Mcmyc.a, pSP64RβG.s, and pSP65RβG.a plasmids (3). Cell extracts were prepared by the method of Manley *et al.* (4). Frog egg extracts were prepared as described (1, 3). dsRNA unwinding activity was assayed *in vitro*. A typical 20-μl reaction mixture contained 10 fmol of ^{32}P-labeled dsRNA, 0.5 mM $MgCl_2$, 50 mM Tris (pH 7.8), 25% glycerol, 50 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, and 5–10 μg of protein extract. After incubation for 2 hr at 37°C, the reaction products were deproteinized and then precipitated with ethanol. RNase treatment with RNase A was carried out as described (3). In some cases, strand separation of sense and antisense RNAs was carried out as described (5).

Base Modification Analysis. dsRNA was prepared from monomer RNAs labeled with [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]CTP, or [α-^{32}P]UTP. The RNA, together with 10 μg of *Escherichia coli* tRNA, was digested either with nuclease P1 into 5′-mononucleotides or with RNase T2 into 3′-mononucleotides. The digests were analyzed by two-dimensional TLC (6). Two solvent systems were used. Solvent system A: isobutyric acid/NH_4OH/water, 100:1.5:48.5 (by volume), in the first dimension and isopropyl alcohol/HCl/water, 70:15:15 (by volume), in the second dimension (7). Solvent system B: isobutyric acid/NH_4OH/water, 66:1:33 (by volume), in the first dimension and 0.1 M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol, 100:60:2 (vol/wt/vol), in the second dimension (8). Determination of the ratio of modified base (A*) to the precursor nucleotide was determined by scraping the radioactive spots off the plates and counting them by liquid scintillation spectroscopy.

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Abbreviations: dsRNA, double-stranded RNA; BAP, bacterial alkaline phosphatase.
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HPLC Analysis. The conditions used for HPLC analysis of base modifications have been described (9–11). β-Globin sense and antisense RNAs were prepared by labeling with [8-^{14}C]ATP. The RNA together with 10 μg of carrier *E. coli* tRNA was digested with nuclease P1 and then with bacterial alkaline phosphatase (BAP). HPLC analysis was carried out using a DuPont Zorbax C_{18} column (25 cm × 4.6 mm, 5- to 6-μm packing) attached to a Perkin–Elmer series 4 liquid chromatograph. Nucleosides were eluted at a flow rate of 0.7 ml/min using a ternary gradient of buffer (0.25 M ammonium acetate, pH 6.0) (A), acetonitrile (B), and water (C) as follows: 0 min, 100% A; 10 min, 96:2:2 (A/B/C); 40 min, 75:10:15; 55 min, 0:40:60. The UV absorbance of the eluate was monitored at 280 nm, and 0.5-min fractions (0.35 ml) were collected for scintillation spectroscopy (11).

RESULTS

The Unwound RNAs Are Structurally Altered and Blocked from Complete Rehybridization. The GM1500 cell extract contains an activity capable of unwinding dsRNA to a form that migrates as a retarded smear after deproteinization on a native polyacrylamide gel (Fig. 1*A*, lane c). The unwound RNA products become sensitive to RNase (Fig. 1, lanes d). We have previously noted that these unwound RNA products cannot completely hybridize back to RNase-resistant dsRNA (3). To examine this finding more carefully, we conducted a series of hybridization studies with strand-separated RNAs. When the unwound products were strand-separated, sense and antisense RNAs did return to monomer positions (Fig. 1*A*, lanes e, g, i, and k), indicating that the unwound RNA was not fully denatured RNA. As expected, monomer RNAs derived from untreated dsRNA hybridized back to an RNase-resistant compact band (Fig. 1*A*, lanes m and n). However, when sense and antisense strands from the unwound RNA were rehybridized together, they annealed back to the RNase-sensitive smear on a native gel (Fig. 1*A*, lanes o and p). Furthermore, when the sense strand from the unwound RNA and antisense strand from the untreated RNA were hybridized, they also formed a smeared complex (Fig. 1*A*, lane q) but one that was less retarded on the gel and less sensitive to RNase (Fig. 1, lanes r). Monomer RNA that was carried through the unwinding assay procedure hybridized to an RNase-resistant compact band (results not shown). From these studies we conclude that the dsRNA is not fully unwound to sense and antisense monomers and that there is a blockage of rehybridization due to some structural alteration of the unwound RNA.

Unwinding Activity Modifies Adenosine Residues. We next analyzed the unwound RNA for base modification. c-*myc* dsRNA was prepared from sense and antisense strands labeled with radioactive ATP, GTP, UTP, or CTP. The unwound RNAs were strand-separated and digested into 5′-mononucleotides (pN) with nuclease P1 or into 3′-mononucleotides (Np) with RNase T2. We found that the P1 digest of the ATP-labeled antisense strand of unwound RNA gave rise to a modified base (A*) as mapped by two-dimensional TLC (Fig. 2*A*, panel b). Antisense RNA derived from untreated dsRNA (Fig. 2*A*, panel a) does not show this modification. P1 digests of [α-^{32}P]GTP-, [α-^{32}P]CTP-, and [α-^{32}P]UTP-labeled RNAs showed no additional modifications (data not shown). Similarly, the sense strand of unwound RNA was found to contain the same adenosine modification (data not shown). A second solvent system was used and again the chromatogram of the P1 digest showed the pA* (Fig. 2*B*, panel d). T2 digests of GTP-, CTP-, and UTP-labeled unwound RNA showed the presence of the A*p, which migrated at the same orientation relative to the Np bases as pA* did relative to pN bases (data not shown). [α-^{32}P]ATP-labeled monomer RNA carried through the unwinding reaction did not acquire the modification (Fig. 2*B*, panel e), suggesting the modification is specific for dsRNA. To confirm this finding, the unwinding reaction mixture was preincubated for 10 min with a 50-fold excess of unlabeled β-globin dsRNA prior to the addition of ^{32}P-labeled c-*myc* dsRNA. We found that this preincubation completely inhib-

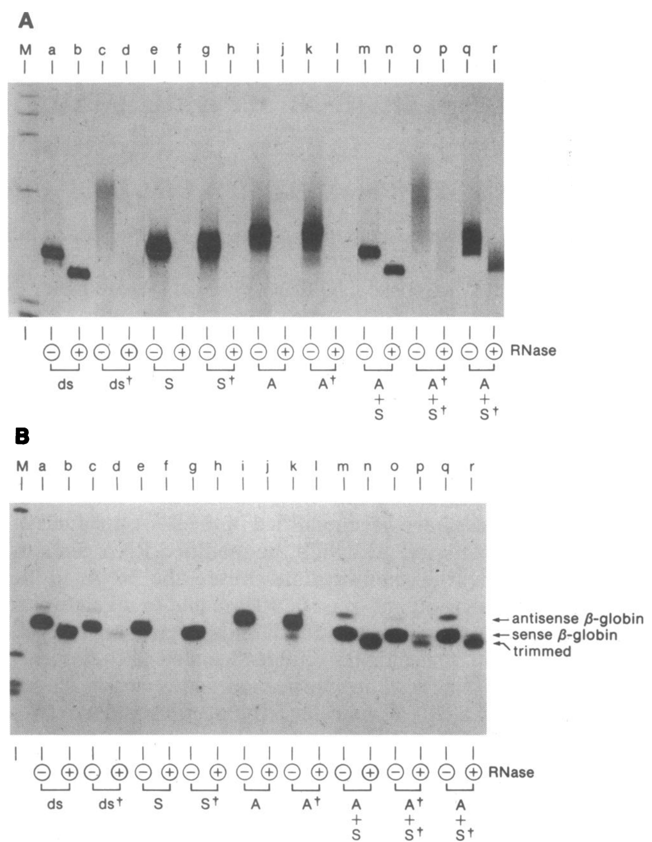

FIG. 1. Unwound RNAs are prohibited from complete rehybridization. Sense β-globin (347 nucleotides) and antisense β-globin (369 nucleotides) RNAs were prepared as described (3). Sense (10^7 cpm/μg) and antisense (10^6 cpm/μg) RNAs were hybridized as a 1:1 molar ratio to form duplex RNA. The duplex RNA with single-stranded overhangs of 8 and 30 nucleotides from the sense and antisense strands, respectively, derived from the polylinker sequences of the pSP64 and pSP65 vectors (lane a), can be trimmed to 339 base pairs (bp) by RNase A (lanes b). The duplex RNA was incubated with the GM1500 cell extract for 2 hr at 37°C (unwound RNA). The duplex RNA was also incubated with GM1500 extract previously digested with proteinase K (untreated RNA). Samples of the "unwound" or "untreated" dsRNA were saved for analysis. The remainder was loaded onto a strand-separating gel. These strand-separated sense and antisense RNAs were then rehybridized overnight at 47.5°C at a 1:1 molar ratio and half of the hybridization mixture was subjected to digestion with RNase A. The samples were electrophoresed on a native (*A*) or denaturing (*B*) 4% polyacrylamide gel. Samples loaded onto the denaturing gel were heat-denatured (95°C, 5 min) in 90% formamide. Lanes a and b, duplex RNA without (−) and with (+) RNase treatment, respectively; lanes c and d, unwound RNA; lanes e and f, strand-separated sense RNA prepared from untreated RNA; lanes g and h, strand-separated sense RNA prepared from unwound RNA; lanes i and j, strand-separated antisense RNA prepared from untreated RNA; lanes k and l, strand-separated antisense RNA prepared from unwound dsRNA; lanes m and n, rehybridized untreated sense and antisense RNAs; lanes o and p, rehybridized unwound sense and antisense RNAs; lanes q and r, unwound sense hybridized to untreated antisense RNA. Lanes M, 5′-end ^{32}P-labeled ΦX174 *Hae* III digests included as size markers. The faint unwound sense RNA band in lane k (*B*) is a contaminant of the strand separation and represents <1% of the total antisense RNA loaded onto the gel. Daggers (†) indicate RNAs derived from unwound RNA.

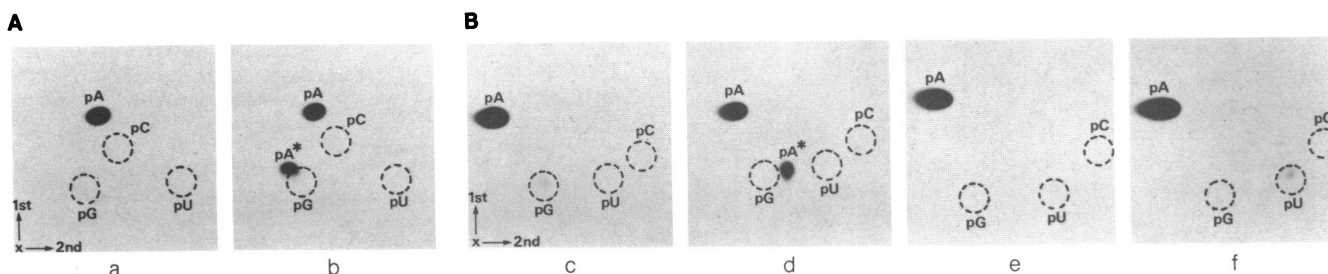

FIG. 2. Modification of adenosine residues occurs during the unwinding reaction. Sense (583 nucleotides) and antisense (605 nucleotides) c-*myc* monomer RNAs were prepared by labeling with [α-^{32}P]ATP, [α-^{32}P]GTP, [α-^{32}P]CTP, or [α-^{32}P]UTP (3). These RNAs were hybridized to form duplex RNA and then were treated with either GM1500 cell extract or the extract previously digested with proteinase K as described in the legend to Fig. 1. With the exception of panel e (which is derived from monomer RNA, see below), these unwound and untreated RNAs were strand-separated. RNA samples were digested into 5'-mononucleotides with nuclease P1 for base modification analysis. The digests were analyzed by two-dimensional TLC using two separate solvent systems (A) and (B) (see text). Only the ATP-labeled RNA showed a base modification. Relative positions of pC, pG, and pU bases are indicated as visualized by UV light. (*A*) Solvent system A, panel a, antisense RNA (10^7 cpm/μg) derived from untreated dsRNA; panel b, antisense RNA derived from unwound RNA. (*B*) Solvent system B, panel c, same as panel a; panel d, same as panel b; panel e, monomer antisense c-*myc* RNA incubated with GM1500 extract for 2 hr; panel f, a 50-fold excess of nonradioactive β-globin dsRNA was preincubated for 10 min at 37°C with GM1500 extract prior to the addition of ^{32}P-labeled c-*myc* dsRNA. In the case of panel f only, c-*myc* dsRNA was prepared from [α-^{32}P]ATP-labeled antisense (10^7 cpm/μg) and [α-^{32}P]UTP-labeled sense (10^7 cpm/μg) RNAs. Therefore the pU contaminant in this case is an indication of the cross-contamination in the strand-separation step, which was $<2\%$.

ited the modification (Fig. 2*B*, panel f). A 50-fold excess of dsDNA, tRNA, or homopolymers poly(dA), poly(dT), poly(rA), poly(rU), or poly(A)·poly(U) did not inhibit the modification (data not shown). The double-stranded polymers poly(A-U)·poly(A-U) and double-stranded reovirus RNA did inhibit the modification, however (data not shown). In the case of the c-*myc* dsRNA, as many as 25% of the adenosine residues are modified using the GM1500 cell extract and present assay conditions.

Adenosine Is Converted to Inosine in the Unwound RNA. In the two solvent systems used, we noted that the modified base ran close to inosine monophosphate on conventional nucleotide maps (12). We therefore added unlabeled pI to P1 digests of the unwound RNA as a standard and reran the samples in solvent systems A and B. We found that in both solvent systems, the pA* base comigrates with the pI standard as viewed by autoradiography and UV shadowing, respectively (data not shown).

To confirm the finding of adenosine conversion to inosine, we also analyzed the unwound RNA by HPLC. HPLC has been used for identification and quantitation of the major and modified nucleoside composition of tRNA, and it resolves inosine from all other known modified adenosines (9–11). The unwound and control untreated RNAs labeled with [8-^{14}C]ATP were prepared. The RNAs were next digested with nuclease P1 and BAP into mononucleosides that were resolved on a C_{18} reverse-phase analytical column. As shown in Fig. 3, 25% of the ^{14}C cpm eluted off the column with the unlabeled inosine standard, and the remainder eluted with adenosine. The control untreated RNA showed only a [^{14}C]-adenosine peak as did monomer RNA that was incubated with the extract (data not shown). From these results, together with the TLC mapping experiments, we conclude that the adenosine modification in the unwound RNA is inosine.

Conversion of Adenosine to Inosine Parallels Disruption of the dsRNA Helix. We next carried out a time course analysis to correlate the inosine modification with the unwinding assay. As the native gel shows, the unwound RNA forms a characteristic smear during the time course (Fig. 4*A*, lanes b–f). In the 8.3 M urea gel system, sense and antisense RNAs (Fig. 4*A*, lane m) migrate differently from dsRNA (Fig. 4*A*, lane g) and unwound RNA (Fig. 4*A*, lanes h–l). As the time course progresses, the smeared RNA complex reaches a plateau (Fig. 4*A*, lanes j and k), which, notably, is not the same as the monomer RNAs (Fig. 4*A*, lane m). Base modification analysis at various time points shows a pattern similar to the smearing effect, where conversion of adenosine to inosine is increased in parallel (Fig. 4*B*, panels b–f). Quantitation of these spots shows a rapid conversion of adenosine to inosine from 0 to 1 hr that levels off from 1 to 2 hr (Fig. 4*C*). From these results, we conclude that the smear of the unwound RNA detected on both a native gel and 8.3 M urea gel directly parallels the accumulation of inosine. This effect is highly indicative of a structural disruption resulting from the modification and subsequent base-pairing mismatch. Furthermore, the 8.3 M urea gel demonstrates once again that the RNA duplex is not unwound fully to monomer RNA but rather remains as an incompletely denatured RNA complex.

Various mammalian cell extracts and frog egg extracts used previously in the detection of the unwinding activity (3) were also tested for the modifying activity. Similar to the time course study, the relative smearing of the unwound RNA pattern by the *Xenopus* egg and mammalian cell extracts from Daudi Burkitt

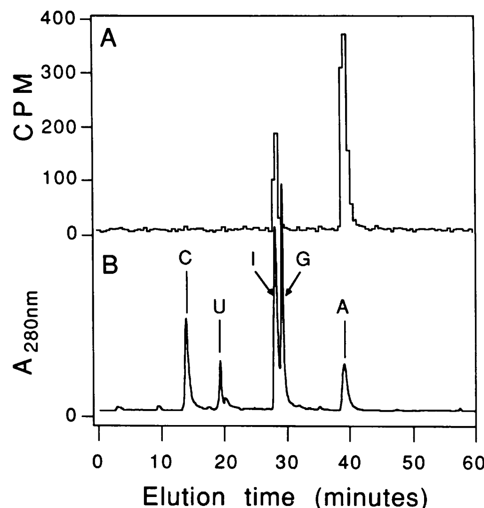

FIG. 3. HPLC analysis of base modification. β-Globin dsRNA was prepared by labeling with [8-^{14}C]ATP. Unwound RNA together with *E. coli* tRNA was digested into mononucleosides using nuclease P1 and BAP. The digest together with inosine (included as an unlabeled internal standard) was fractionated with a C_{18} reverse-phase analytical HPLC column. (*A*) Radioactivity of each 0.5-min fraction plotted as cpm vs. time. (*B*) UV absorbance of the eluate, monitored at 280 nm, plotted vs. time. The elution time of the inosine standard was separately confirmed by a control scan of a 10-fold excess of inosine vs. guanosine. Similarly, the elution times of m^{1}A (25 min) and xanthosine (24 min) were confirmed (not shown).

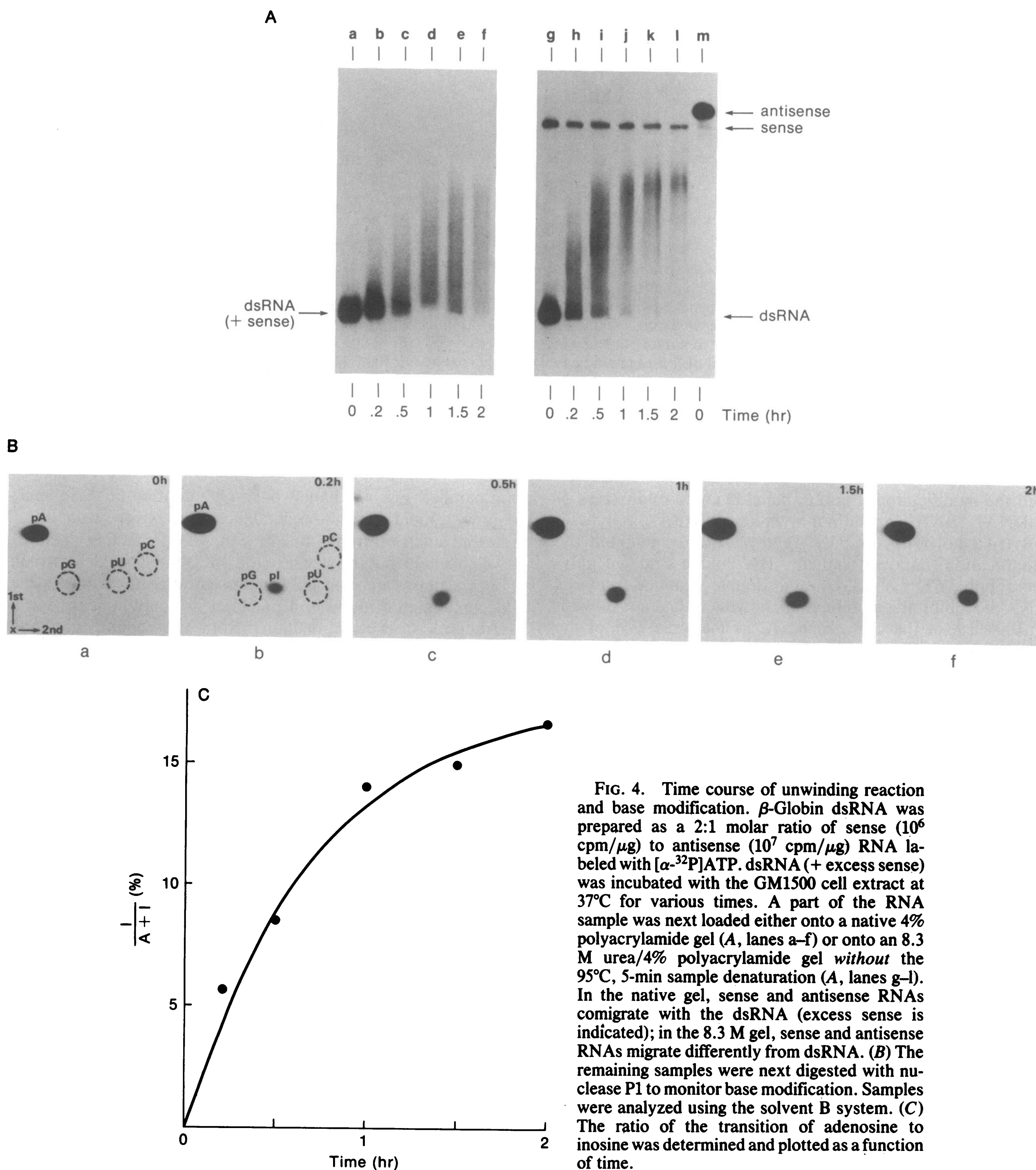

FIG. 4. Time course of unwinding reaction and base modification. β-Globin dsRNA was prepared as a 2:1 molar ratio of sense (10^6 cpm/μg) to antisense (10^7 cpm/μg) RNA labeled with [α-^{32}P]ATP. dsRNA (+ excess sense) was incubated with the GM1500 cell extract at 37°C for various times. A part of the RNA sample was next loaded either onto a native 4% polyacrylamide gel (*A*, lanes a–f) or onto an 8.3 M urea/4% polyacrylamide gel *without* the 95°C, 5-min sample denaturation (*A*, lanes g–l). In the native gel, sense and antisense RNAs comigrate with the dsRNA (excess sense is indicated); in the 8.3 M gel, sense and antisense RNAs migrate differently from dsRNA. (*B*) The remaining samples were next digested with nuclease P1 to monitor base modification. Samples were analyzed using the solvent B system. (*C*) The ratio of the transition of adenosine to inosine was determined and plotted as a function of time.

lymphoma, mouse plasmacytoma P3xAg8, and mouse NIH 3T3 fibroblast directly paralleled the amount of adenosine to inosine conversion (results not shown). The greatest was from the *Xenopus* egg extract, in which 40% of the adenosine residues were converted to inosine in the dsRNA. As noted previously (3), unwinding/modifying activity was low in NIH 3T3 cells synchronized into quiescence and increased in cells stimulated by fetal calf serum (data not shown).

DISCUSSION

A Modifying Activity and the Structure of the Modified RNA. Our results in this and in our previous report (3) clearly indicate that there is an activity in many different cell types and species that changes the secondary structure of dsRNA to a completely different form. The altered dsRNA has undergone a 25–40% conversion of adenosine to inosine. We have examined the nearest neighbor analysis for the inosine in the unwound RNA (6) and have found that the modification has the following 3′-neighbor preference G = C ≥ U > A, suggesting that there may be sequence selectiveness of the modification (R.W.W. and K.N., unpublished results). The adenosine to inosine conversion results in a mismatched I-U base pair that presumably adopts a wobble hydrogen bonding configuration (13). It is this series of I-U mismatches that causes the RNA to migrate anomalously on a native poly-

acrylamide gel. The I-U mismatch probably exposes the U base to nucleophilic attack by RNase A, an enzyme known to cleave mispaired pyrimidines (14).

The unwinding/modifying activity is very specific to the dsRNA. Intermolecular dsRNA rather than intramolecular dsRNA appears to be the substrate for the activity. For instance, tRNA as well as monomer RNA, which presumably contain short intramolecular dsRNA regions, are not substrates. Presently, we have not checked the lower size limit of intermolecular dsRNA; the shortest duplex to be modified is a 198-bp portion of the c-*myc* dsRNA (R.W.W. and K.N., unpublished results). Interestingly, partial dsRNA with 5′ and 3′ overhangs of single-stranded RNA as well as blunt-ended dsRNA are modified equally well by this activity (R.W.W. and K.N., unpublished results).

As we have previously described, the unwinding/modifying activity is likely to be different from any other already known unwinding activities (3). In addition, RNA duplex unwinding activity that results in an accompanying base modification of the substrate RNA has not been reported previously to our knowledge. Several enzymes involved in purine metabolism are known to catalyze the conversion of adenine to hypoxanthine (the free base of inosine) by way of deamination at the N6 position of the adenine ring. However, such enzymes, including adenosine deaminase and 5′-adenylic acid deaminase, are known to have strict substrate specificities (15). We have tested the commercially available adenosine deaminase and 5′-adenylic acid deaminase (Sigma) and have found that they do not modify adenosine in β-globin dsRNA (unpublished results).

Implications for Antisense RNA Approach. Our initial interest in the dsRNA unwinding activity was initiated by the fact that certain antisense-transformed cells, which expressed high levels of antisense RNA, did not exhibit phenotypic changes. This unwinding activity was proposed to render the sense RNA available for translation even in the presence of antisense RNA. The results of the present study contradict this conclusion for the following reasons. We have conclusively shown that the RNA products are not fully unwound monomer strands as previously proposed (1, 2). If the sense strand of the unwound RNA became available *in vivo* for translation, possibly by another unwinding activity, it may not be accurately translated since 25–40% of adenosine residues have been changed to inosine residues. In conclusion, the unwinding/modifying activity may in fact enhance the effect of antisense RNA blockage and may not be a cause of the failure of some antisense RNA experiments.

The Biological Role of dsRNA Modification. One of our most plausible hypotheses for the physiological role of the unwinding/modifying activity is that the activity may be involved in a mechanism that degrades duplex RNA formed *in vivo*. Intermolecular networks of RNA that contain duplex regions have been reported in developing frog embryos (16) and human lymphoblastoid cells (17). It is possible that these dsRNAs may be more quickly degraded by ribonucleases after introduction of I-U base mismatches. Another candidate as a possible substrate for unwinding/modifying activity is naturally occurring sense-antisense RNA duplexes from eukaryotic genes. Antisense RNA has been identified in the regulation of diverse and complex phenomena in prokaryotes (19). Several examples of antisense RNA transcription of eukaryotic genes have been reported (20–23). The unwinding/modifying activity might be involved in modulating the RNA duplex formed between such naturally occurring sense and antisense RNAs as a part of a regulatory mechanism as yet unidentified in eukaryotes.

It should be noted that the unwinding/modifying activity could alter the expression of certain mRNAs posttranscriptionally by converting adenosine to inosine and consequently changing their coding capacity. Examples of posttranscriptional modification or editing of mRNAs have been reported (24–27). Interestingly, in the matrix gene of a heavily mutated measles virus, 50% of the U residues were changed to C residues (18). Although it was hypothesized that this highly biased mutational rate may be due to a transient, defective RNA polymerase (18), these mutations could be introduced by the unwinding/modifying activity described in this report (B. Bass and H. Weintraub, personal communication). The reported mutations of U to C in the plus strand can be copied from the minus strand previously modified by A to I conversions. If, however, such mutations are indeed introduced into genomes of RNA viruses through the unwinding/modifying activity, it is still not clear whether introduction of these mutations is a part of a cellular defense system against viral infection or whether viruses subvert unwinding/modifying activities, intended for other cellular mechanisms, and thereby gain rapid mutations.

Note. After completion of our studies presented in this report, we learned that Bass and Weintraub had also obtained results essentially identical to ours (28).

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