MOLECULAR AND CELLULAR BIOLOGY, Feb. 1988, p. 770–777
0270-7306/88/020770-08$02.00/0

Vol. 8, No. 2

Cell Cycle Expression of RNA Duplex Unwindase Activity in Mammalian Cells

RICHARD W. WAGNER AND KAZUKO NISHIKURA*

The Wistar Institute, 36th Street at Spruce, Philadelphia, Pennsylvania 19104

Received 28 September 1987/Accepted 23 November 1987

An RNA duplex unwindase activity has been found by using an in vitro assay with various types of mammalian, somatic cells, including HeLa, mouse plasmacytoma, and Burkitt lymphoma. The unwindase activity is very low in mouse fibroblast 3T3 cells arrested into quiescence, but increases when the cells are released into renewed growth by serum. In addition, a gel retardation assay proved to be specific and sensitive for detection of RNA duplex-unwindase complexes.

A series of experiments has shown that introduction of an antisense RNA complementary to a specific mRNA into cells can effectively create or mimic the null-mutant phenotype, which is quite useful for investigating gene products of unknown physiological function (4, 5, 8, 10–13, 17, 25). Using this antisense RNA approach, we were also able to show that c-*fos* nuclear proto-oncogene expression is a prerequisite for the transition from G_0 into renewed cell growth (21). During the course of these previous studies, we noticed that the c-*fos* mRNA levels of antisense c-*fos*-transformed clones were not decreased in the continuously proliferating state. In the same clones, however, the endogenous c-*fos* gene expression was greatly decreased during the transition from quiescence into the early G_1 period, suggesting that the efficiency of the antisense RNA approach varies with some specific states of the cell cycle. In addition, we also obtained antisense c-*myc* RNA-transformed clones from various cell lines, including mouse fibroblast NIH 3T3 and mouse Friend leukemia cells. However, despite the integration of a large number of antisense c-*myc* DNA sequences fused to the mouse mammary tumor virus or methallothionine promoter, these clones failed to exhibit phenotypic changes.

Recently, two independent groups reported that *Xenopus laevis* eggs and early embryos contain an activity capable of specifically dissociating RNA duplexes (1, 24), which explained the failure of the antisense RNA approach with early amphibian embryos. These findings led us to examine whether a similar unwinding activity can also be found in mammalian, somatic cell systems, especially where the application of the antisense RNA approach was unsuccessful. By using an in vitro assay with double-stranded RNA (dsRNA) and crude extracts from various cells, we found that a similar unwindase activity is indeed present in various types of mammalian cells including HeLa, human lymphoblastoid, mouse plasmacytoma, Burkitt lymphoma, and mouse F9 teratocarcinoma cells. The mammalian unwindase activity is specific to dsRNA and identical to that of *Xenopus* embryos except that it has an optimum temperature of 37°C instead of 25°C.

In mouse fibroblast 3T3 cells arrested into quiescence, the level of unwindase activity is very low, but it increases when the cells are stimulated by fetal calf serum (FCS). Thus the RNA unwindase gene, similar to those for dihydrofolate reductase (7), thymidine kinase (28), or histone (9), which exhibits a transient increase in expression during the cell cycle, may be responding to a cell cycle-specific regulatory signal.

Using a gel retardation assay, we have detected a protein(s) which binds specifically to the RNA duplex. Interaction between the protein and RNA duplex is reversible. Although this is not yet proven, the binding protein detected is most likely unwindase itself. This fast-binding assay system may facilitate biochemical purification of unwindase activities from mammalian tissue culture cell extracts.

MATERIALS AND METHODS

Chemicals and materials. *Xenopus laevis* (South African clawed toad) was purchased from Xenopus I (Ann Arbor, Mich.). SP6 polymerase and RNasin were purchased from Promega Biotec (Madison, Wis.); RNase A (type IIIA), RNase T_1 (grade IV), mare serum, and human chorionic gonadotropins were obtained from Sigma Chemical Corp. (St. Louis, Mo.); ribonucleotides and cap analogs came from Pharmacia-PL Biochemicals (Piscataway, N.J.); vanadyl-ribonucleoside complexes were obtained from Bethesda Research Laboratories (Gaithersburg, Md.); and radionucleotides were obtained from Amersham Corp. (Arlington Heights, Ill.).

Plasmid construction. The plasmids used for preparation of sense and antisense RNAs were constructed as follows: pSP64Mcmyc.s and pSP65Mcmyc.a were constructed from a mouse c-*myc* cDNA clone, pMcmyc54 (27). A portion of the cDNA, the *Hin*dIII-*Pst*I fragment, consisting of a large part of the first exon (423 base pairs [bp]) and 134 bp of the second exon, was excised from pMcmyc54 and then religated into the *Hin*dIII and *Pst*I sites of pSP64 or pSP65 vector (18). In the pSP64Mcmyc.s plasmid, the orientation of the mouse c-*myc* gene is the same as the SP6 transcription, whereas it is the opposite in pSP65Mcmyc.a. pSP64RβG.s and pSP65RβG.a were constructed from a recombinant plasmid, psβ, carrying a part of the rabbit β-globin gene and simian virus 40 sequences (A. Buchman and P. Berg, unpublished data) (22). The *Hin*dIII-*Bam*HI DNA fragment, consisting of the first (135 bp) and second (339 bp) exons of the rabbit β-globin gene, was excised from psβ and then inserted into the *Hin*dIII and *Bam*HI sites of the pSP64 or pSP65 vector. In pSP64RβG.s, the orientation of the rabbit β-globin

* Corresponding author.

gene is the same as the SP6 transcription, whereas it is the opposite in pSP65RβG.a.

RNA synthesis and preparation of duplex. Capped sense and antisense RNAs were transcribed in vitro from pSP64Mcmyc.s (sense), pSP65Mcmyc.a (antisense), pSP64RβG.s (sense), and pSP65RβG.a (antisense) plasmids using SP6 polymerase according to the method described previously (1, 18). The reaction contained 40 mM Tris (pH 7.4), 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each of ATP, CTP, GTP, and UTP, 2.5 mM GpppG, 40 U of SP6 polymerase, 60 U of RNasin, and 3 μg of plasmid DNA that had been linearized with the appropriate restriction enzyme. [α-^{32}P]UTP, 200 or 20 μCi, was included to label antisense or sense RNA, respectively. The reaction was carried out at 37°C, and the template DNA was removed by subsequent DNase digestion (18). The amount of RNA synthesized was calculated on the basis of counts per minute of [α-^{32}P]UTP incorporated.

Sense (10^6 cpm/μg) and antisense (10^7 cpm/μg) RNAs were hydbridized at a 1:1 ratio in 80% formamide–40 mM PIPES [piperazine-*N*,N′-bis(2-ethanesulfonic acid), pH 6.7]–0.4 M NaCl–1 mM EDTA at 47.5°C for 12 h after a 5-min denaturation at 95°C, and the RNA duplex was further purified as described previously (24). The duplex was suspended in 20 mM NaCl–10 mM Tris (pH 7.5). Mouse c-*myc* transcripts were 583 nucleotides (sense) and 605 nucleotides (antisense) in length. The RNA duplex formed between the sense and antisense c-*myc* RNA has 8-nucleotide (sense) and 30-nucleotide (antisense) overhangs derived from the polylinker sequences of pSP64 and pSP65 vectors. RNases trim these overhangs, resulting in a shorter (575 nucleotides) duplex (see Fig. 1A). Rabbit β-globin transcripts were 347 nucleotides (sense) and 369 nucleotides (antisense), and the duplex had 8-nucleotide (sense) and 30-nucleotide (antisense) overhangs. With RNase treatment, the RNA duplex became 339 nucleotides in length (Fig. 1B).

Cell culture. Burkitt lymphoma Daudi cell line and human lymphoblastoid cell line GM1500 OUB/6TG (20) were grown in RPMI 1640 medium supplemented with 10% FCS. HeLa cells obtained from R. Weinmann were grown in minimal essential medium with 10% FCS. Mouse plasmacytoma P3xAg8, obtained from W. Gerhard, and mouse embryonic teratocarcinoma F9, obtained from C. Howe, were both grown in Dulbecco modified Eagle medium with 10% FCS. Mouse fibroblast BALB/c 3T3 cells obtained from the American Type Culture Collection were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum.

Cell extract preparation. Eggs of *X. laevis* were prepared from females that had been injected with gonadotropin 8 to 12 h earlier, and the eggs were decapsulated for 2 min in a solution of 2% (wt/vol) L-cysteine neutralized to pH 7.8 with 0.1 N NaOH (19). S100 extracts of *Xenopus* eggs were prepared according to the method described previously (1). Extracts from somatic cells were prepared by the method of Manley et al., described previously (16), with a minor modification. The final cell extracts were dialyzed against a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 0.1 M KCl, 0.1 mM EDTA, 2 mM dithiothreitol, 17% glycerol, and 2 mM $MgCl_2$ instead of 12.5 mM. Small samples of the S100 and Manley extracts were stored at −70°C after rapid freezing in liquid N_2. The protein concentration in extracts was determined by Lowry protein assay and averaged 5 to 10 mg/ml.

All suspension cells were grown in a culture flask with a stirring bar to exponential growth and were then harvested for extraction. F9 and BALB/c 3T3 cells were grown in square (22 by 22 cm) culture dishes and harvested when they were approximately 50% confluent. To obtain extracts from cells at a specific point of the cell cycle, BALB/c 3T3 cells were grown to confluency for 7 days, after which time the medium was replaced with low-serum medium containing 0.5% calf serum. After 2 days of incubation in this serum-deprived condition, which synchronizes cells into a quiescent state, the cells were stimulated by fresh medium containing 10% FCS and incubated for various times.

RNA duplex unwindase assay and analysis of RNA. RNA duplex unwindase activity (RNA denaturase) of various cell extracts was assayed in vitro (1). Unless specified, the reaction was carried out in 20 μl and contained 5 fmol of ^{32}P-labeled dsRNA, 0.5 mM $MgCl_2$, 50 mM Tris (pH 7.8), 25% glycerol, 50 mM KCl, 0.1 mM EDTA, and 0.5 mM dithiothreitol. The reaction was stopped by adding 200 μl of 1-mg/ml proteinase K in 50 mM Tris (pH 7.5)–5 mM EDTA–0.5% sodium dodecyl sulfate, and the sample was incubated for 30 min at 37°C, extracted with phenol and chloroform, and ethanol precipitated. The samples were electrophoresed in a native 4% polyacrylamide gel as described previously (1), and in some cases, half of each sample was electrophoresed in a denaturing 7 M urea–4% polyacrylamide gel.

RNase treatment. ^{32}P-labeled RNAs were treated in 200 μl of ice-cold 0.3 M NaCl–10 mM Tris (pH 7.5)–5 mM EDTA containing 2 μg of RNase T_1 and 40 μg of RNase A per ml for 2 h at 15°C, and 200 μl of 2-mg/ml proteinase K in 0.1 M Tris (pH 7.5)–10 mM EDTA–1% sodium dodecyl sulfate was added to the reaction products. After 30 min of incubation at 37°C, the digestion products were extracted with phenol and chloroform and then ethanol precipitated.

RNA mobility shift binding assay. ^{32}P-labeled c-*myc* RNA duplex (50 fmol) was incubated in standard unwindase reaction mix (20 μl) with GM1500 cell extract containing various amounts of protein at 30°C for 10 min. One half of the reaction mixture (10 μl) was mixed with 2 μl of loading buffer (50 mM Tris-glycine [pH 8.8], 20% glycerol, 0.05% xylene cyanole, 0.05% bromophenol blue) and loaded directly onto a 4% polyacrylamide–50 mM Tris-glycine (pH 8.8) gel. Electrophoresis was carried out at room temperature as described previously (14). For competition binding assays, a 50- to 150-fold excess (relative to RNA duplex) of various reagents or competitors was pre- or postincubated with GM1500 cell extracts for 10 min at 30°C before or after the addition of ^{32}P-labeled c-*myc* RNA duplex.

Autoradiography. All autoradiography was carried out with a fluorescent screen and preflashed X-ray film at −70°C (15).

RESULTS

Unwindase activity in various mammalian cells. An in vitro assay system (1) was used to investigate RNA duplex unwindase activity. As a model gene, the mouse mini c-*myc* gene (575 bp), which consists of a part of the first and second exons cloned into pSP64 and pSP65 vectors (18), was used (Fig. 1A). Capped sense (583 nucleotides) and antisense (605 nucleotides) c-*myc* RNAs were synthesized in vitro using SP6 polymerase (18) (Fig. 1A). During in vitro transcription, these RNAs were labeled with [α-^{32}P]UTP, but antisense RNAs had 10 to 20 times higher specific radioactivity than sense RNAs. These "hot" antisense and "warm" sense c-*myc* RNAs were hybridized in vitro, and the resultant RNA duplex was used as a substrate for an in vitro RNA duplex unwindase assay. We confirmed that *X. laevis* egg

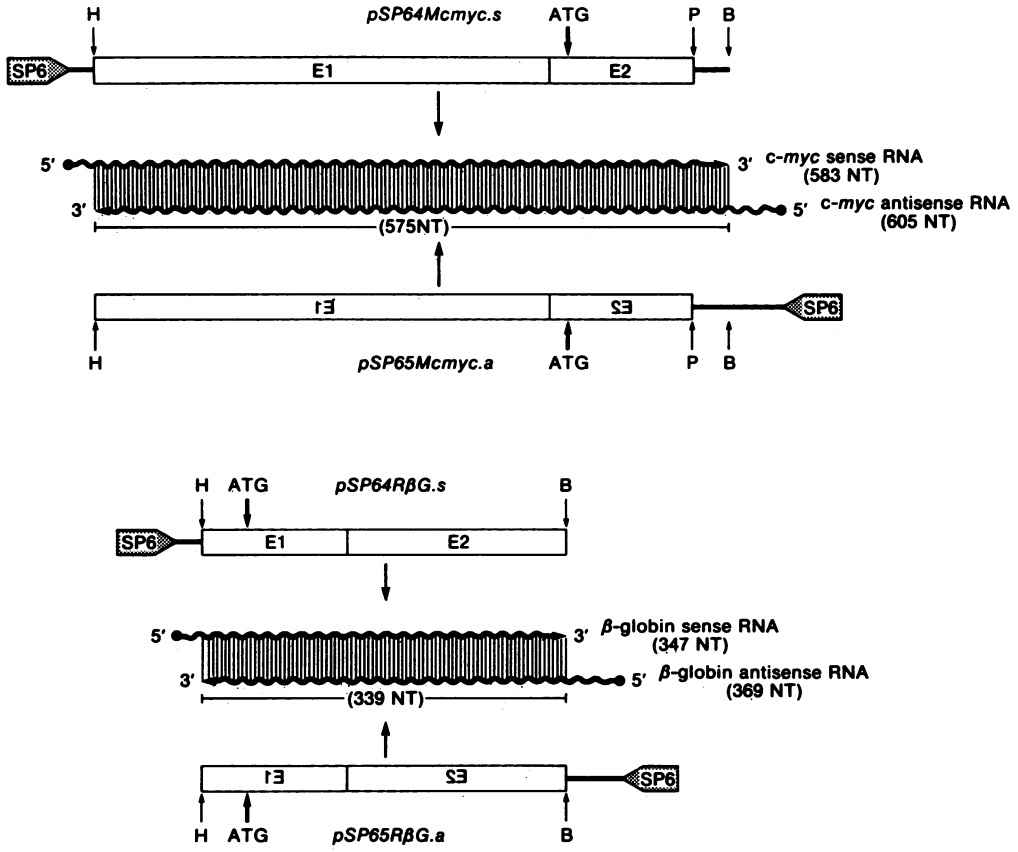

FIG. 1. Schematic representation of sense and antisense RNA synthesis and duplex preparation. The stippled boxes mark the SP6 promoters. Open boxes represent exons. Solid lines are a part of the polylinker sequences derived from either pSP64 or pSP65 vectors. The location of the translation initiation codon ATG of the c-*myc* gene and β-globin gene is also indicated. Restriction sites: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I. Wavy lines and arrows represent the SP6 transcripts and orientations. Fine vertical lines between sense and antisense RNAs indicate a complementary portion of the RNA duplex formed. Sense and antisense RNA duplexes contain single-stranded overhangs derived from the polylinker sequences of pSP64 and pSP65 vectors, and these overhangs can be digested by RNases A and T_1. (Top) Synthesis of mouse sense and antisense c-*myc* RNAs and formation of the c-*myc* RNA duplex. The pSP64Mcmyc.s plasmid, linearized with *Bam*HI, and the pSP65Mcmyc.a plasmid, linearized with *Hin*dIII, give 583-nucleotide sense c-*myc* and 605-nucleotide antisense c-*myc* transcripts, respectively. (Bottom) Synthesis of rabbit β-globin sense and antisense RNAs and formation of the β-globin RNA duplex. The pSP64RβG.s plasmid, linearized with *Bam*HI, and the pSP65RβG.a plasmid, linearized with *Hin*dIII, give 347-nucleotide sense β-globin and 369-nucleotide antisense β-globin transcripts, respectively.

extracts contain an activity which unwinds this mouse c-*myc* RNA duplex. The migration pattern of ^{32}P-labeled c-*myc* duplex (Fig. 2A, lane e) changed on a native gel after incubation with cell extracts at 25°C for 3 h (lane g). The unwound nature of the reaction products was confirmed by their susceptibility to RNase treatment (Fig. 2A and B, lanes h). The same RNase treatment did not degrade the intact duplex, although it trimmed the single-stranded overhangs of the duplex (Fig. 2A and B, lanes f), thus shortening it (see Fig. 1A). However, the unwound RNAs must be structurally different from both duplex and monomer forms of sense and antisense RNAs, since both duplex and monomer RNAs migrated to locations much lower and more compact than the products of the unwindase reaction in the native gel (Fig. 2A, lanes a, c, e, and g). Sense and antisense monomer c-*myc* RNAs were both sensitive to RNase treatment, as expected (Fig. 2A, lanes b and d; Fig. 2B, lanes b and d). We confirmed a very similar result on another RNA duplex formed between in vitro-synthesized sense and antisense rabbit β-globin RNAs (see Fig. 2C). The duplex could be unwound by incubation with the *Xenopus* egg extracts, and the products migrated differently from the duplex or monomers (Fig. 2C, lanes a, c, e, and g). From these results, we conclude that in vitro-made mouse c-*myc* or rabbit β-globin RNA duplexes can be unwound by an activity in *Xenopus* egg extracts. However, it appears that the duplex is not only being unwound, but also is becoming modified to a form which is clearly different from the original monomer form as judged by its migration pattern on a native gel.

We next investigated whether the RNA unwindase activity could be found in mammalian cells other than amphibian eggs and embryos. We found (Fig. 3) that the same activity was present in various mammalian tissue culture cell lines such as HeLa, human lymphoblastoid (GM1500), and mouse plasmacytoma (P3xAg8). In addition to these cell lines, we found that human Burkitt lymphoma (Daudi) and mouse embryonic teratocarcinoma F9 cells also contained a similar unwindase activity (data not shown).

Mammalian RNA unwindase activity seems to have an optimum temperature higher than that of amphibian RNA unwindase (Fig. 3), although the reaction products of unwindase from *Xenopus* eggs and mammalian cells appear to be identical (Fig. 4A and B). The size of unwound ^{32}P-labeled mouse c-*myc* RNAs treated with egg (Fig. 4, lanes g), HeLa

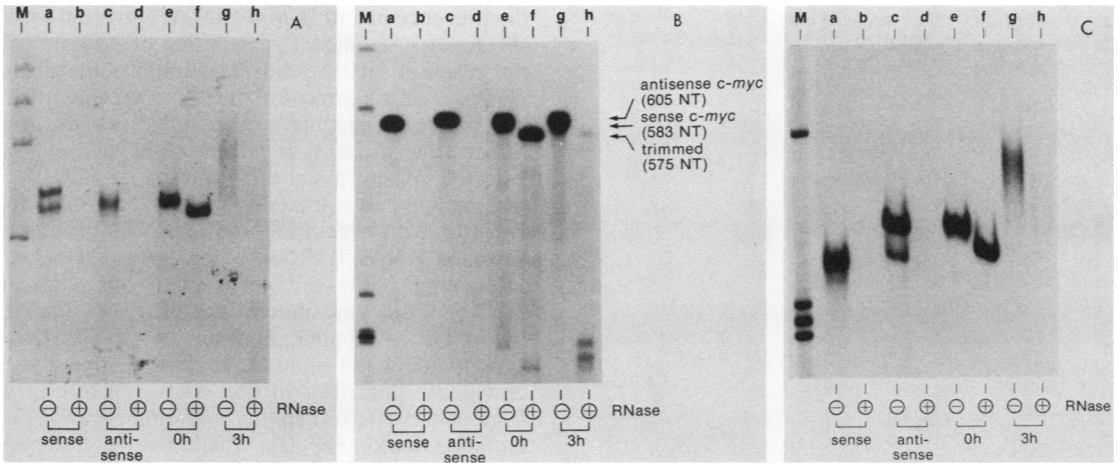

FIG. 2. c-*myc* and β-globin RNA duplexes are unwound by the activity present in *Xenopus* egg extracts. RNA duplex was mixed with an S100 extract of *Xenopus* eggs containing 40 μg of protein and 10 mM $MgCl_2$ for 3 h or 0 h of incubation in 20 μl of reaction mixture at 25°C. RNA was deproteinized, and then half of the sample was treated with RNases A and T_1. All RNA samples were electrophoresed in a native 4% polyacrylamide gel (A and C) or in a denaturing 7 M urea–4% polyacrylamide gel (B). Sense (lanes a and b) and antisense (lanes c and d) monomer RNAs without (−) or with (+) RNase treatment were also analyzed. (A and B) Sense and antisense c-*myc* RNAs and the duplex were analyzed. It was noted that monomer RNA appeared as a doublet band on a native gel (A, lane a), although it migrated to the location expected from its size as a single band on a denaturing gel (B, lane a). We therefore attribute the doublet to two different secondary structures of the sense monomer RNA. NT, Nucleotide. (C) Sense and antisense β-globin RNAs and the duplex were analyzed. Lanes M, 5′-end ^{32}P-labeled φX174 *Hae*III digests included as size markers in each autoradiogram.

(lanes i), and GM1500 (lanes k) cell extracts was unchanged on a denaturing gel, and these reaction products were equally susceptible to RNase treatment (lanes h, j, and l). From these results, we conclude that the RNA duplex unwindase activity can be found in a wide variety of mammalian cells.

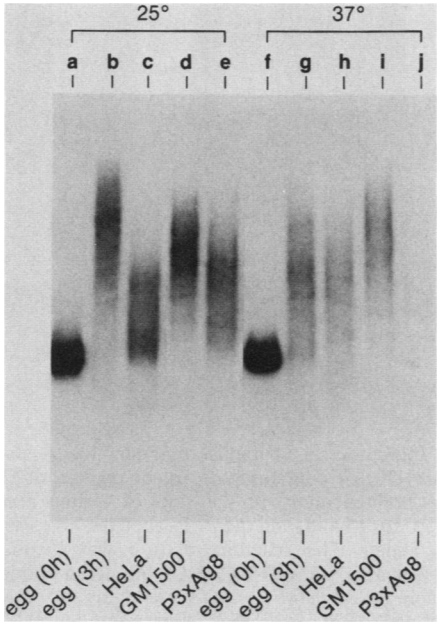

FIG. 3. RNA unwindase activity is also present in a variety of mammalian cells. Duplex c-*myc* RNA was incubated with an S100 extract of *Xenopus* eggs or a Manley extract of various cells containing 40 μg of protein and 10 mM $MgCl_2$ for 3 h at either 25 or 37°C. RNA was deproteinized and analyzed in a native 4% polyacrylamide gel. As a control, the duplex was incubated with S100 egg extract which had been treated with proteinase K at 37°C for 30 min before the addition of the duplex (lanes a and f).

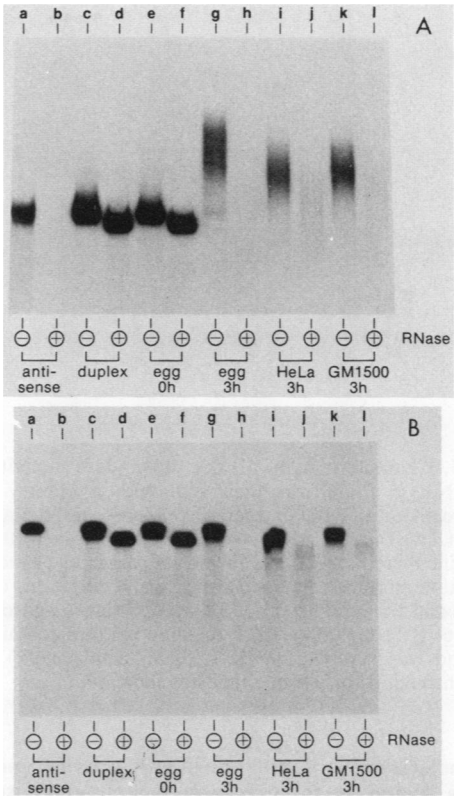

FIG. 4. Identical reaction products of *Xenopus* and mammalian unwindase. Duplex c-*myc* RNA (5 fmol) was incubated either with an S100 extract of *Xenopus* eggs at 25°C for 0 h (lanes e and f) or 3 h (lanes g and h) or with a Manley extract of HeLa or GM1500 cells at 37°C for 3 h (lanes i through l). RNA was deproteinized, and then half of the sample was treated with RNases A and T_1. All RNA samples were electrophoresed in a native 4% polyacrylamide gel (A) and also in a denaturing 7 M urea–4% polyacrylamide gel (B). As controls, antisense c-*myc* monomer RNAs (lanes a and b) and duplex RNAs (lanes c and d) were also analyzed.

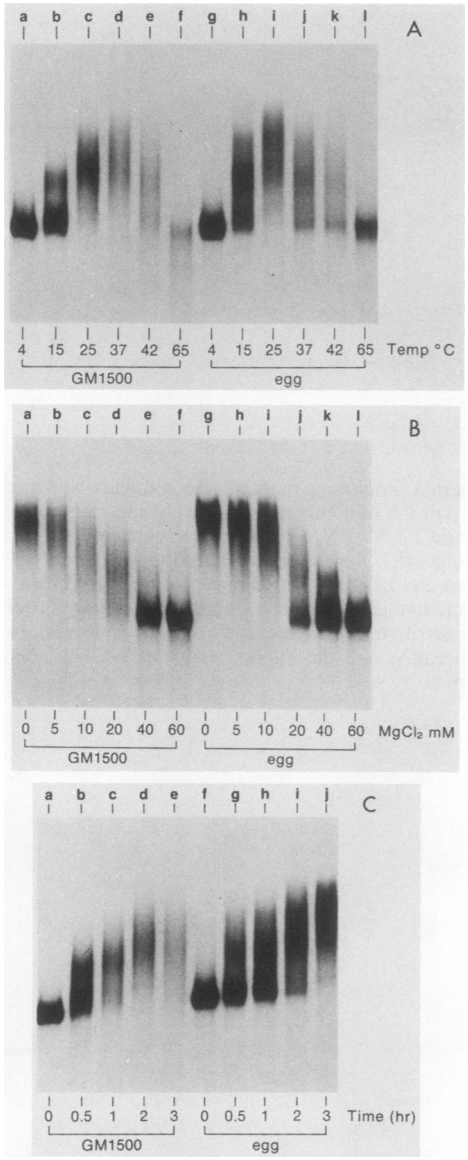

FIG. 5. Characterization of the unwindase activity. Duplex c-*myc* RNA (5 fmol) was incubated with a Manley extract of GM1500 cells or an S100 extract of *Xenopus* eggs containing 40 μg of protein. (A) The reaction mixture in 20 μl contained 10 mM $MgCl_2$ and was incubated for 3 h at various temperatures as indicated. (B) The reaction mixture contained various concentrations of $MgCl_2$ as indicated and was incubated for 3 h at 37°C (lanes a through f) or at 25°C (lanes g through l). (C) The reaction mixture contained 10 mM $MgCl_2$ and was incubated at 37°C (lanes a through e) or at 25°C (lanes f through j) for various times as indicated.

Characterization of the unwindase activity and reaction products. Using the in vitro assay system and extracts from *Xenopus* eggs and human lymphoblastoid cell line GM1500, optimum conditions for amphibian and mammalian RNA duplex unwindase activities were compared. Unwindase from GM1500 cell extracts has an optimum temperature of 37°C, while the unwinding activity from frog eggs is optimal at 25°C (Fig. 5A). We found that the unwindase activity from mammalian cells as well as frog eggs does not require any additional Mg^{2+} in the assay mixture (Fig. 5B). Nonrequirement of Mg^{2+} for unwindase reaction was also confirmed in the presence of 1 to 10 mM EDTA, which, in fact, stimulated the activity significantly. Addition of ATP up to 200 μM to the reaction mixture did not stimulate the mammalian unwindase, as reported for the *Xenopus* unwindase (1) (data not shown). The time course suggests that unwinding is a rather slow process, requiring more than 3 h to complete with the current assay conditions (Fig. 5C). The activity in mammalian cell extracts was specifically inhibited by preincubating with excess (50-fold) dsRNAs but not with single-stranded RNAs (ssRNAs), dsDNAs, ssDNAs, or tRNA. Interestingly, the double-stranded homopolymer poly(A) · poly(U) did not inhibit the activity. Cap precursors, m^7GpppG or GpppG, also did not inhibit the unwindase activity (data not shown), suggesting that the mammalian unwindase described in this study is likely to be different from eucaryotic translation initiation factors. For example, initiation factor eIF-4F, which has ATP-dependent RNA unwinding activity, is inhibited by cap precursors (23). In addition, we found that vanadyl-ribonucleoside complexes (known RNase inhibitors) (2) strongly inhibited the unwindase, whereas another RNase inhibitor, RNasin (26), did not (Fig. 6B).

Since the smeared unwindase products on the native gels appeared to be incompletely dissociated forms of the RNA duplex, we carried out additional experiments to understand the nature of this effect. Initially, we hypothesized that either the fully unwound products (monomers) always par-

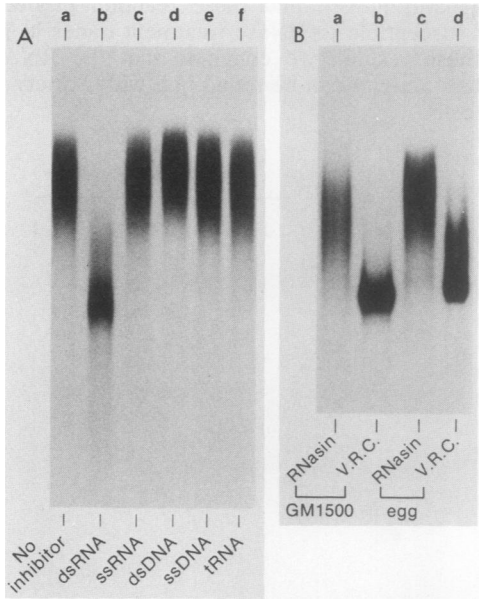

FIG. 6. Inhibition of unwindase reaction. (A) A standard reaction mixture (20 μl) containing 40 μg of protein of GM1500 cell extract was preincubated with 250 fmol of various reagents (unlabeled) at 37°C for 15 min, and then ^{32}P-labeled c-*myc* RNA duplex (5 fmol) was added. The reaction mixture was incubated for an additional 2 h at 37°C. dsRNA, β-Globin RNA duplex; ssRNA, sense β-globin RNA; dsDNA, dsDNA carrying the *Hin*dIII-*Bam*HI fragment of pSP64RβG.s; ssDNA, single-stranded M13 DNA from mp10cfosM.s; tRNA, yeast tRNA. (B) Duplex c-*myc* RNA (5 fmol) was incubated for 2 h with cell extract containing 40 μg of protein from GM1500 cells at 37°C (lanes a and b) or with cell extract from *Xenopus* eggs at 25°C (lanes c and d). The reaction mixture contained 100 U of RNasin (lanes a and c) or 10 mM vanadyl-ribonucleoside complexes (lanes b and d). After deproteinization, the RNA samples were electrophoresed in a native 4% polyacrylamide gel.

tially renatured upon gel loading or the unwindase could not fully denature the RNA into monomer forms. To test the former possibility, both monomers of sense and antisense RNAs were reacted together with GM1500 cell extract under standard unwindase assay conditions. When the deproteinized reaction products (i.e., proteinase K treated, followed by phenol-chloroform extraction) were electrophoresed on a native gel, the RNAs continued to migrate as monomer forms (confirmed by RNase susceptibility) with no evidence of the smeared pattern seen in the case of the RNA duplex (results not shown).

Since the monomers did not hybridize in the reaction or during gel loading, we next examined the denaturing capacity of the unwindase itself. Longer incubation of the standard reaction with excess protein, or inclusion of additional protein into the assay after 2 h, or incubation of deproteinized assay products with fresh extract for 2 h did not alter the migration pattern of the RNA on a native gel as compared with the standard assay products (results not shown). However, treatment of deproteinized reaction products with 90% formamide at 90°C for 3 min before loading on a native gel fully denatured the products to monomer forms (results not shown). We were therefore led to conclude that the unwindase may not fully denature the RNA duplex. In addition to these experiments, we subjected deproteinized reaction product RNAs to hybridization protocols (excluding the initial 95°C heating; see Materials and Methods) at either 47.5 or 37°C. To our surprise, the smeared migration pattern of the RNA still did not change, suggesting that the unwound products cannot rehybridize back to duplex form (results now shown). The reaction products may be somehow modified to prevent renaturation. We conclude that the migration pattern of unwindase reaction products differs from those of both monomer and duplex RNAs on a native gel because of these novel forms of incompletely unwound products.

Cell cycle expression of the unwindase activity in 3T3 cells. Since various mammalian cell lines containing a high level of unwindase activity are very actively proliferating cells, we next investigated the possibility of involvement of this activity in the proliferation mechanism. Cell extracts were prepared from BALB/c 3T3 mouse fibroblast cells that had either been synchronized into quiescence, stimulated with FCS for various times, or exponentially grown without synchronization. Using these cell extracts, we found that very little, if any, of the activity was present in the quiescent 3T3 cells (Fig. 7, lanes e and f). However, after stimulation of quiescent cells by FCS, the activity increased in cells stimulated for 6 to 12 h (lanes i through l) and slightly decreased in cells stimulated for 24 h (lanes m and n). We also found that continuously proliferating 3T3 cells contained an intermediate level of the activity (lanes o and p). Mixing of the extracts from quiescent 3T3 cells and from the cells stimulated for 12 h with 10% FCS did not decrease the unwindase activity of the latter alone (data not shown). This result indicates that the low level of the unwindase activity in the quiescent 3T3 cells is not due to the presence of an inhibitor in this resting state of the cell cycle. From these results, we conclude that the RNA duplex unwindase activity found in mammalian cultured cells originating from various tissues increases transiently during the cell cycle.

Detection of RNA duplex-unwindase complexes. We next examined the possibility of detecting the RNA duplex-unwindase complexes by a gel retardation assay, using the low-ionic-strength, native polyacrylamide gel electrophoresis system used recently for detection of RNA spliceosome

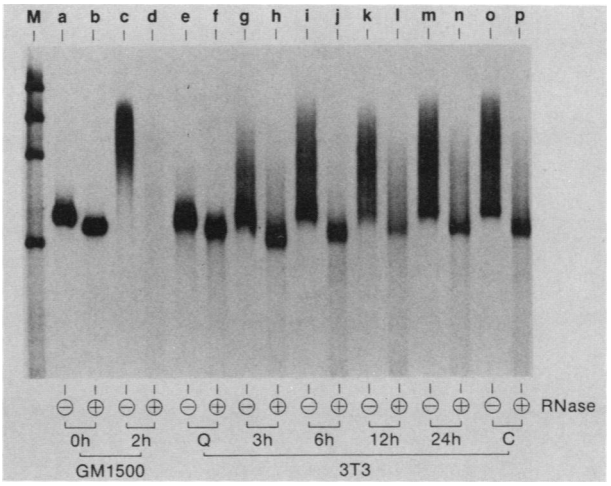

FIG. 7. Cell cycle expression of unwindase activity in mouse fibroblast 3T3 cells. Duplex c-*myc* RNA (5 fmol) was incubated with cell extract containing 40 μg of protein from GM1500 (lanes a through d) or BALB/c 3T3 fibroblast cells (lanes e through p) at 37°C for 0 h (lanes a and b) or 2 h (lanes c through p). The GM1500 cell extract was prepared from exponentially growing cells, whereas the 3T3 cell extract was prepared from cells in various stages of the cell cycle. Q, Quiescent 3T3 cells; C, exponentially growing 3T3 cells; 3, 6, 12, or 24 h, quiescent 3T3 cells stimulated with 10% FCS for 3, 6, 12, or 24 h. After incubation, RNA was deproteinized, and then half of each sample was treated with RNases A and T_1 (+). The relative activity of unwindase in different cell extracts can be easily determined by comparing the intensity of the duplex band remaining intact after RNase treatment: the more the activity, the less the duplex remained (see, for example, lanes d and f). All RNA samples were electrophoresed in a native 4% polyacrylamide gel. M, ^{32}P-labeled, φX174 *Hae*III digests.

complexes (14). Incubation of 50 fmol of RNA duplex with increasing amounts of extract protein from GM1500 cells formed a titration curve in which the smeared complex saturated at 24 μg of protein (Fig. 8A, lane e). Due to the time and temperature of the reaction (10 min at 30°C), smearing was attributed to an initial binding of cell extract protein and not to the unwindase reaction (data not shown). To investigate the specificity of the protein-RNA interaction, several potential inhibitors/competitors also used for the unwindase reaction assay were tested. Preincubation with excess (100-fold) RNA duplex completely inhibited the binding of this protein in the ^{32}P-labeled RNA duplex (Fig. 8B, lane e), whereas preincubation with the same concentration of ssRNA (lane f), dsDNA (lane g), ssDNA, poly(A) · poly(U) homopolymer, or tRNA (data not shown) did not affect formation of complexes. Incubation of RNA duplex with the extract showed that RNA duplex mobility remained unchanged at low pH (Fig. 8C, lane b). In addition, a postincubation with excess (50-fold) RNA duplex (Fig. 8C, lane d) but not dsDNA (lane e) fully disrupted the RNA duplex-protein complex, suggesting that the interaction is specific and reversible. Additional evidence has shown that binding is also not reversed by ssRNA, tRNA, poly(A) · poly(U), or ssDNA (results not shown). We obtained similar gel retardation results with *Xenopus* egg extracts. We conclude that the specific binding of a protein(s), likely unwindase itself although not yet proven, to the RNA duplex can be detected as an RNA duplex mobility shift on a native polyacrylamide gel. The binding appeared to be reversible.

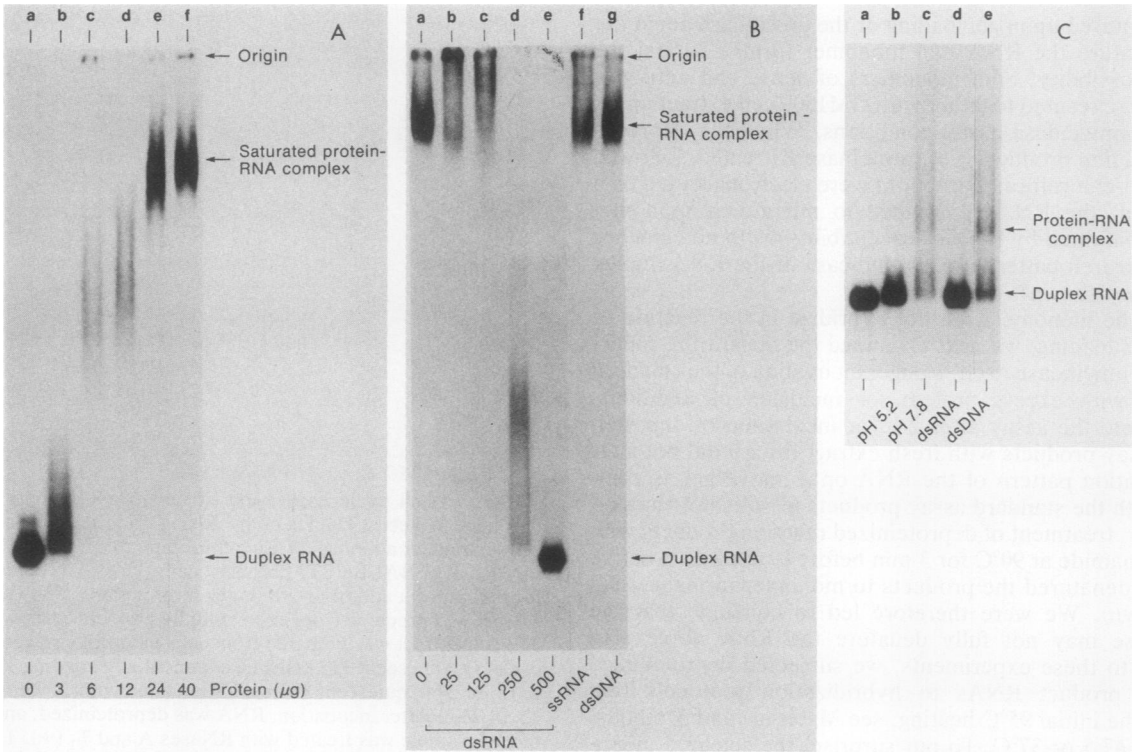

FIG. 8. Gel retardation and competition assay for RNA duplex-unwindase complexes. (A) ^{32}P-labeled c-*myc* RNA duplex (50 fmol) was incubated with increasing amounts of GM1500 cell extract for 10 min at 30°C before electrophoresis in a 4% polyacrylamide gel with a Tris-glycine buffer (pH 8.8) (see Materials and Methods). (B) Various amounts of unlabeled dsRNA (rabbit β-globin) (lanes a through e), ssRNA (sense rabbit β-globin) (lane f), or dsDNA (lane g) were preincubated with 10 μg of GM1500 cell extract for 10 min at 30°C, followed by an addition of 5 fmol of ^{32}P-labeled RNA duplex (mouse c-*myc*). The reaction was incubated for a further 30 min at 30°C before electrophoresis. Lanes: a, no competitor; b, 25 fmol of dsRNA; c, 125 fmol of dsRNA; d, 250 fmol of dsRNA; e, 500 fmol of dsRNA; f, 500 fmol of ssRNA; g, 500 fmol of dsDNA. (C) ^{32}P-labeled duplex RNA (2.5 fmol) was incubated with 0.7 μg of GM1500 cell extract for 20 min at 30°C before loading onto a retardation gel. Lane a, Naked RNA duplex in the absence of protein. Lanes b and c, Results of incubation of protein and RNA at pH 5.2 and at pH 7.8, respectively. Lanes d and e, Protein was incubated with the ^{32}P-labeled c-*myc* RNA duplex at pH 7.8 for 10 min, followed by a 10-min "cold" chase with 125 fmol (50-fold excess) of dsRNA (rabbit β-globin RNA) or dsDNA (rabbit β-globin DNA), respectively.

DISCUSSION

In this study, we have shown that an RNA duplex unwindase activity is present in crude cell extracts from a variety of mammalian tissue culture cell lines. As determined by an in vitro assay, this activity appears to be identical to that recently reported in *X. laevis* eggs or early embryos (1, 24) except that it has a slightly higher optimum temperature. The activity is specifically inhibited by dsRNA, but not by ssRNA, tRNA, dsDNA, ssDNA, or poly(A) · poly(U) homopolymer. These findings suggest that the unwindase activity is specific to dsRNA. The activity is also inhibited by one type of RNase inhibitor, vanadyl-ribonucleoside complexes, whereas another RNase inhibitor, RNasin, does not affect the unwindase activity. The products of unwindase reaction are susceptible to RNases A and T_1, yet appear to be different from original monomer or single-stranded RNAs on a native polyacrylamide gel. The products also appear to be modified and somehow prohibited from renaturation, even after deproteinization.

We have found that the level of unwindase activity in mouse fibroblast 3T3 cells changes depending on their state within the proliferative cycle. Cells arrested into quiescence appeared to have less activity than serum-stimulated cells or continuously proliferating cells. In addition, our preliminary results suggest that the unwindase activity decreases when mouse embryonic F9 stem cells are induced with retinoic acid and dibutyryl cyclic AMP to differentiate into endoderm cells (data not shown). It is known that in vitro-induced differentiation parallels the decline in proliferative activity (6). Interestingly, a large fraction of the poly(A)$^+$ RNA from human lymphoblastoid cells, in which we have found a high level of unwindase activity, was reported to contain repetitive sequences that form intermolecular networks when annealed in vitro (3). Cells which transcribe RNAs carrying repetitive sequences may contain more unwindase activity to keep the mRNAs in free, dissociated forms. The level of the RNAs carrying repetitive sequences and thus the level of the unwindase activity may also vary within a cell depending on the state of cell cycle or differentiation.

It is noteworthy that antisense c-*fos* RNA expressed in 3T3 cells can effectively lower the sense c-*fos* mRNA and protein levels and subsequently block the entry of the cells into S phase (21). A sharp rise in c-*fos* gene expression takes place within a very short period after mitogen stimulation of quiescent 3T3 cells (i.e., perhaps before the increase in unwindase activity), and this may be the reason why the antisense RNA approach was effective in our previous study (21). Thus, although the antisense RNA seems to be a very useful tool to investigate vertebrate or mammalian genes with unknown physiological functions (10, 17), it may not work efficiently in some cells or specific states of the cells in which high levels of unwindase activities are present.

We have also shown here that binding of a protein(s) to the RNA duplex can be detected by a gel retardation assay using a low-ionic-strength, native polyacrylamide gel electrophoresis system. The result of the titration experiment with increasing amount of extract proteins has indicated that the protein(s) binds at multiple sites, which appears to saturate the RNA duplex. In addition, the competition assay revealed that the binding is specific to dsRNA and that interaction between the protein(s) and the RNA duplex is reversible. These results suggest that the protein(s) detected most likely includes the RNA duplex unwindase. Interestingly, binding of the unwindase to the RNA duplex appeared to be complete within a rather short time (10 min) and even at low temperature (4°C, data not shown), whereas by comparison the unwinding of the RNA duplex is rather slow, taking more than 3 h at 37°C to complete under the present assay conditions. The gel retardation assay of the RNA duplex-protein complexes can be used to facilitate biochemical purification of this unwindase protein(s). The biochemical and structural nature of RNA duplex-unwindase complexes is currently being investigated.

ACKNOWLEDGMENTS

We thank R. P. Ricciardi and J. M. Murray for critical reading of the manuscript. We also thank W. Pilgermyer and U. Kim for expert technical assistance and the Editorial Services Department of the Wistar Institute for preparing this manuscript.

This project was supported by the National Science Foundation (grant DCB-8511103 to K.N.) and the American Cancer Society (grant JFRA-122 to K.N.). R.W. was supported by a Public Health Service training grant (5-T32-HD07152) from the National Institutes of Health.

LITERATURE CITED

1. **Bass, B. L., and H. Weintraub.** 1987. A developmentally regulated activity that unwinds RNA duplexes. Cell **48**:607–613.
2. **Berger, S. L., and C. S. Birkenmeier.** 1979. Inhibition of intractable nucleases with ribonucleoside-vanadyl complexes: isolation of messenger ribonucleic acid from resting lymphocytes. Biochemistry **18**:5143–5149.
3. **Calabretta, B., D. L. Robberson, A. L. Maizel, and G. F. Saunders.** 1981. mRNA in human cells contains sequences complementary to the Alu family of repeated DNA. Proc. Natl. Acad. Sci. USA **78**:6003–6007.
4. **Coleman, J., P. J. Green, and M. Inouye.** 1984. The use of RNAs complementary to specific mRNAs to regulate the expression of individual bacterial genes. Cell **37**:429–436.
5. **Crowley, T. E., W. Nellen, R. H. Gormer, and R. A. Firtel.** 1985. Phenocopy of discoidin I-minus mutants by antisense transformation in Dictyostelium. Cell **43**:633–641.
6. **Dean, M., R. A. Levine, and J. Campisi.** 1986. c-*myc* regulation during retinoic acid-induced differentiation of F9 cells is post-transcriptional and associated with growth arrest. Mol. Cell. Biol. **6**:518–524.
7. **Farnham, P. J., and R. T. Schimke.** 1985. Transcriptional regulation of mouse dihydrofolate reductase in the cell cycle. J. Biol. Chem. **260**:7675–7680.
8. **Green, P. J., O. Pines, and M. Inouye.** 1986. The role of antisense RNA in gene regulation. Annu. Rev. Biochem. **55**: 569–597.
9. **Hentschel, C., and M. L. Birnstiel.** 1981. The organization and expression of histone gene families. Cell **225**:301–313.
10. **Izant, J. G., and H. Weintraub.** 1984. Inhibition of thymidine kinase gene expression by antisense RNA: a molecular approach to genetic analysis. Cell **36**:1007–1015.
11. **Izant, J. G., and H. Weintraub.** 1985. Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA. Science **229**:345–352.
12. **Kim, S. K., and B. J. Wold.** 1985. Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA. Cell **42**:129–138.
13. **Knecht, D. A., and W. F. Loomis.** 1987. Antisense RNA inactivation of myosin heavy chain gene expression in Dictyostelium discoideum. Science **236**:1081–1086.
14. **Konarska, M. M., and P. A. Sharp.** 1987. Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. Cell **49**:763–774.
15. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335–341.
16. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. USA **77**:3855–3859.
17. **Melton, D. A.** 1985. Injected antisense RNAs specifically block messenger RNA translation in vivo. Proc. Natl. Acad. Sci. USA **82**:144–148.
18. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
19. **Newport, J., and M. Kirschner.** 1982. A major developmental transition in early Xenopus embryos. I. Characterization and timing of cellular changes at the midblastula stage. Cell **30**: 675–686.
20. **Nishikura, K., J. Erikson, A. ar-Rushdi, K. Huebner, and C. M. Croce.** 1985. The translocated c-*myc* oncogene of Raji Burkitt lymphoma cells is not expressed in human lymphoblastoid cells. Proc. Natl. Acad. Sci. USA **82**:2900–2904.
21. **Nishikura, K., and J. M. Murray.** 1987. Antisense RNA of proto-oncogene c-*fos* blocks renewed growth of quiescent 3T3 cells. Mol. Cell. Biol. **7**:639–649.
22. **Nishikura, K., and G. A. Vuocolo.** 1984. Synthesis of two mRNAs by utilization of alternate polyadenylation sites: expression of SV40-mouse immunoglobulin μ chain gene recombinants in Cos monkey cells. EMBO J. **3**:689–699.
23. **Ray, B. K., T. G. Lawson, J. C. Kramer, M. H. Cladaras, J. A. Grifo, R. D. Abramson, W. C. Merrick, and R. E. Thach.** 1985. ATP-dependent unwinding of messenger RNA structure by eukaryotic initiation factors. J. Biol. Chem. **260**:7651–7658.
24. **Rebagliati, M. R., and D. A. Melton.** 1987. Antisense RNA injections in fertilized frog eggs reveal an RNA duplex unwinding activity. Cell **48**:599–605.
25. **Rosenberg, U. B., A. Preiss, E. Seifert, A. Jaeckle, and D. C. Knipple.** 1985. Production of phenocopies by Krüppel antisense RNA injection into Drosophila embryo. Nature (London) **313**: 703–706.
26. **Scheele, G., and P. Blackburn.** 1979. Role of mammalian RNAase inhibitor in cell-free protein synthesis. Proc. Natl. Acad. Sci. USA **76**:4898–4902.
27. **Stanton, L. W., R. Watt, and K. B. Marcu.** 1983. Translocation, breakage and truncated transcripts of c-*myc* oncogene in murine plasmacytomas. Nature (London) **303**:401–406.
28. **Thompson, C. B., P. B. Challoner, P. E. Neiman, and M. Groudine.** 1985. Levels of c-*myc* oncogene mRNA are invarient throughout the cell cycle. Nature (London) **314**:363–366.