Role of Poly(A) Polymerase in the Cleavage and Polyadenylation of mRNA Precursor

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To determine the role of poly(A) polymerase in 3'-end processing of mRNA, the effect of purified poly(A) polymerase antibodies on endonucleolytic cleavage and polyadenylation was studied in HeLa nuclear extracts, using adenovirus L3 pre-mRNA as the substrate. Both Mg^{2+} - and Mn^{2+} -dependent reactions catalyzing addition of 200 to 250 and 400 to 800 adenylic acid residues, respectively, were inhibited by the antibodies, which suggested that the two reactions were catalyzed by the same enzyme. Anti-poly(A) polymerase antibodies also inhibited the cleavage reaction when the reaction was coupled or chemically uncoupled with polyadenylation. These antibodies also prevented formation of specific complexes between the RNA substrate and components of nuclear extracts during cleavage or polyadenylation, with the concurrent appearance of another, antibody-specific complex. These studies demonstrate that (i) previously characterized poly(A) polymerase is the enzyme responsible for addition of the poly(A) tract at the correct cleavage site and probably for the elongation of poly(A) chains and (ii) the coupling of these two 3'-end processing reactions appears to result from the potential requirement of poly(A) polymerase for the cleavage reaction. The results suggest that the specific endonuclease is associated with poly(A) polymerase in a functional complex.

The 3' termini of most functional eucaryotic mRNAs are generated by specific endonucleolytic cleavage of the precursor molecules, followed by the sequential addition of a poly(A) tract containing 200 to 300 adenylate residues (for a recent review, see reference 20). With the development of cell-free systems (19, 28) which generally use HeLa nuclear extracts capable of accurate and efficient polyadenylation of pre-mRNAs, significant progress has been made toward understanding the biochemistry of this unique posttranscriptional modification. For example, these systems have facilitated identification of *cis*-acting sequences in the precursor molecule which are essential for the 3'-end processing of pre-mRNAs. Cleavage and polyadenylation depend on at least two sequences, a highly conserved hexanucleotide, located 10 to 30 nucleotides upstream of the poly(A) addition site of most mRNAs (13, 21, 31, 44, 47), and less conserved, downstream G+U- or U-rich sequences (4, 8, 9, 11, 22-24, 36, 39). The latter sequences do not appear to be indispensable for polyadenylation but rather modulate the efficiency of usage of poly(A) sites. An additional element, a dinucleotide, is the site of poly(A) addition, the second nucleotide of which is usually an adenosine residue to which adenylate residues are added (2, 24, 29).

Contrary to the progress made in identification of nucleotide sequences essential for polyadenylation, the factors that interact with these functionally important sequences have not been isolated and characterized. Recently, there has been some success in the fractionation of nuclear extracts into two components, one containing poly(A) polymerase and another containing the endonuclease activity (43). A more recent study with fractionated HeLa nuclear extracts has suggested the involvement of small nuclear RNA(s) in the cleavage reaction on the basis of sensitivity to micrococcal nuclease and to α -sm monoclonal antibody (10). Since these fractions have not been extensively purified, the nature of the interaction between various components in the cleav-

age-polyadenylation reaction is not evident. It is also unknown whether other components are involved in the 3'-end processing of mRNA. One way to approach this problem is to purify the functional components and use them in a reconstitution assay. Alternatively, antibodies specific for each component can be used in an *in vitro* system to study the exact roles of the components in the cleavage-polyadenylation reaction.

Our laboratory has extensively characterized nuclear poly(A) polymerase from rat liver and a rat hepatoma (34, 41). The liver enzyme is structurally and immunologically distinct from the corresponding tumor enzyme (41, 42). We have obtained polyclonal antibodies which, upon Western blot (immunoblot) analysis, exhibit a single band corresponding to the 48-kilodalton (kDa) tumor poly(A) polymerase in a relatively crude nuclear extract (41). The specificity of these antibodies has been further demonstrated by the lack of their reaction with purified liver poly(A) polymerase or with RNA polymerase. This study was undertaken to determine the direct role of poly(A) polymerase in addition of the poly(A) tract to the 3' terminus of mRNA. For this purpose, the effect of antitumor poly(A) polymerase antibodies on the cleavage and polyadenylation of adenovirus L3 mRNA in HeLa nuclear extracts was studied.

MATERIALS AND METHODS

Preparation of pre-mRNA substrates. Radiolabeled transcripts containing the poly(A) site of the adenovirus type 2 L3 transcription unit were produced by SP6-directed transcription of *Dra*I-digested plasmid SP64L3 (a generous gift from Claire Moore). *In vitro* transcription was performed at 40°C for 1 h essentially as described previously (26). The dinucleotide 7meGpppG (final concentration, 1 mM) was included to produce capped precursor RNA (17). The concentrations of ribonucleoside triphosphates added to the reaction were 0.5 mM ATP and CTP, 0.3 mM GTP, 0.1 to 0.25 mM UTP, and 50 μ Ci of [α - 32 P]UTP. Full-length transcripts were purified by electrophoresis on 6% poly-

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acrylamide-7 M urea gels and eluted from the gel slices with a buffer containing 0.5 M ammonium acetate, 0.1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and 1 μ g of carrier tRNA. The RNA was then stored in water at -20°C until used.

In vitro polyadenylation. Nuclear extracts were prepared from HeLa cells by the method of Dignam et al. (5) except that phenylmethylsulfonyl fluoride was omitted from all buffers. Polyadenylation reactions were carried out in a final volume of 25 μ l and contained 32 or 40% HeLa nuclear extract, 2×10^4 to 7×10^4 cpm of α - ^{32}P -labeled precursor RNA, 1 mM ATP, 20 mM creatine phosphate, 0.5 mM MgCl_2 , 2% polyvinyl alcohol, 0.25 mM dithiothreitol, and 40 ng of tRNA per μ l. Accurate cleavage of the 267-nucleotide-long pre-mRNA yields a 218-nucleotide-long product 5' to the poly(A) site. For assays of manganese-dependent poly(A) polymerase activities, MgCl_2 was replaced with 0.5 mM MnCl_2 . Conditions for cleavage reactions were identical to those for polyadenylation reactions except that either 1 mM cordycepin triphosphate (3'-dATP) (ATP omitted) or 2 mM EDTA (MgCl_2 omitted) was included. Reactions performed at 30°C for the indicated times were stopped by the addition of 7 volumes of a buffer containing 100 mM Tris hydrochloride (pH 8.0), 20 mM EDTA, 20 mM NaCl, 0.4% SDS, and proteinase K (final concentration, 200 μ g/ml), followed by incubation for 15 min at 30°C in this buffer. RNA was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and precipitated with ethanol. RNA was then analyzed by electrophoresis on 8% polyacrylamide-7 mM urea gels, followed by autoradiography.

Gel shift analysis of in vitro polyadenylation reactions. Samples (25 μ l) for the processing reactions were incubated under conditions optimal for either polyadenylation or cleavage. Each sample was split into two 12.5- μ l portions. To analyze the RNA products generated in the reaction, one portion was incubated in the proteinase K stop buffer, phenol extracted, ethanol precipitated, and electrophoresed on a denaturing 8% polyacrylamide-7 M urea gel. The second portion was subjected to gel electrophoresis under nondenaturing conditions to allow determination of the nature of specific complexes formed between pre-mRNA and extract components. In this case, the reaction was terminated by the addition of heparin to a final concentration of 5 mg/ml. After a 10-min incubation on ice, 2 μ l of 30% glycerol containing 25% bromophenol blue and 25% xylene cyanol was added, and the samples were resolved by electrophoresis in 4% nondenaturing gels essentially as described previously (18). Gels were preelectrophoresed for at least 45 min, and electrophoresis was then carried out at 4°C and 18 V/cm until the xylene cyanol dye had migrated approximately 20 cm. Both the gel (30 cm long, 1.5 mm thick; acrylamide- N,N' -methylene bisacrylamide ratio, 80:1) and running buffer contained $0.5 \times$ TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). Gels were transferred to 3MM paper (Whatman, Inc., Clifton, N. J.) and autoradiographed at -40°C with a screen.

Production of anti-poly(A) polymerase antibodies. Nuclear poly(A) polymerase was purified essentially to homogeneity from isolated nuclei of transplanted Morris hepatoma 3924A cells as described elsewhere (41). Briefly, nuclear extracts were subjected to sequential column chromatography on DEAE-Sephadex, phosphocellulose, hydroxylapatite, QAE-Sephadex, and DNA-cellulose. To ensure the purity of the antigen sample, the DNA-cellulose poly(A) polymerase was separated on a 10% SDS-polyacrylamide gel before use. The antihepatoma poly(A) polymerase polyclonal antibodies

were raised in male New Zealand White rabbits by biweekly injections with gel slices containing 50 μ g of the purified enzyme mixed with Freund incomplete adjuvant. Antisera were prepared after whole-blood samples were allowed to clot for 15 min at 4°C and stored at -20°C in small portions. For antibody inhibition studies, immunoglobulin G (IgG) purified by DEAE Affi-Gel blue chromatography (Bio-Rad Laboratories, Richmond, Calif.) of the sera followed by ammonium sulfate precipitation was used. Preimmune IgG used in controls was also prepared in this manner, using the sera obtained from the rabbits before immunization.

Immunoblot analysis of HeLa nuclear extracts. Crude nuclear extracts from hepatoma 3924A and HeLa cells were precipitated with equal volumes of acetone on ice for several hours. The dried protein pellets were suspended in buffer, heated for 2 min at 90°C , and subjected to electrophoresis on a 10% SDS-polyacrylamide gel. The proteins were transferred by electrophoresis to nitrocellulose (Hybond C; Amersham Corp., Arlington Heights, Ill.) at 100 mA for 16 h at room temperature in a buffer containing 25 mM Tris hydrochloride, 192 mM glycine, and 20% methanol. The nitrocellulose blots were then blocked in phosphate-buffered saline (PBS) plus 5% powdered milk for several hours at 4°C and probed with antihepatoma poly(A) polymerase antisera (1:50 dilution in PBS plus milk) for 12 to 16 h at 4°C . The blots were then extensively washed (five times for 5 min each) with PBS and incubated with a secondary biotinylated goat anti-rabbit IgG (1:400 dilution) in PBS plus milk at room temperature for 2 h. After additional PBS washes, the blots were incubated for 30 min at room temperature with avidin DH-biotinylated horseradish peroxidase H reagents of the Vectastain ABC kit (Vector Laboratories). After a final series of PBS washes, the blots were incubated in 100 ml of a PBS solution containing 60 mg of 4-chloro-1-naphthol dissolved in methanol and 60 μ l of 30% hydrogen peroxide, which permitted visual detection of immune complexes.

RESULTS

Antibodies raised against purified poly(A) polymerase from Morris hepatoma cells react specifically with a corresponding polypeptide in HeLa nuclear extracts. Immunoblot analysis was performed by using antihepatoma poly(A) polymerase antisera and HeLa nuclear extracts (Fig. 1). When the proteins of HeLa nuclear extracts were first separated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose paper, and then probed with antisera raised against the hepatoma poly(A) polymerase, a distinct immune complex corresponding to a molecular size of 48 kDa was observed (lane 2). A similar immune complex was observed with proteins of hepatoma 3924A nuclear extracts (lane 1). Pre-immune rabbit sera did not react with either the hepatoma or the HeLa nuclear proteins (lanes 3 and 4). These results demonstrate that anti-poly(A) polymerase antibodies directed against the nuclear poly(A) polymerase from hepatoma 3924A cells also reacted with a protein of identical molecular weight present in HeLa nuclear extracts, which indicates that nuclear poly(A) polymerases from hepatoma and HeLa cells are immunologically related. Furthermore, purified IgG could inhibit the poly(A) polymerase activity of HeLa nuclear extracts (data not shown). It should be emphasized that the minor two bands above the 48-kDa band were also occasionally seen with the preimmune sera. These bands appeared to be artifacts produced with the biotinylated probes and were not seen with ^{125}I -protein A probe (41).

FIG. 1. Immunoblot analysis of HeLa crude nuclear extracts with antihepatoma poly(A) polymerase antisera. Nuclear extracts (20 μ g of protein) derived from Morris hepatoma 3924A (lanes 1 and 3) and HeLa (lanes 2 and 4) cells were subjected to electrophoresis under denaturing conditions and transferred electrophoretically to nitrocellulose as described in Materials and Methods. The nitrocellulose blots were then incubated with antihepatoma poly(A) polymerase antisera (lanes 1 and 2) or with preimmune rabbit sera (lanes 3 and 4). A biotinylated secondary antibody protocol was used to allow visual detection of the immune complexes. Lane M, Biotinylated molecular size protein standards; the corresponding approximate molecular size (in kilodaltons) of each standard is indicated on the left. The position corresponding to the molecular size of denatured nuclear poly(A) polymerase (48-kDa) is designated by the arrow.

Antihepatoma poly(A) polymerase antibodies inhibit polyadenylation of adenovirus type 2 L3 precursor mRNA in HeLa nuclear lysates. To determine whether the protein in HeLa nuclear extracts which cross-reacted immunologically with the antihepatoma poly(A) polymerase antibodies might be the enzyme responsible for the addition of poly(A) to the 3' ends of mRNA precursor, the effects of direct addition of these antibodies to a soluble *in vitro* polyadenylation system were tested (Fig. 2). Pre-mRNA substrates which contained the adenovirus L3 poly(A) site were generated by transcription of a *Dra*I-digested SP6 template. The gel-purified, 32 P-labeled, 5'-capped precursor RNA (267 nucleotides in length) was then incubated on ice for 30 min in HeLa nuclear extracts that had been pretreated with various amounts of IgG purified from antihepatoma poly(A) polymerase antisera. These samples were incubated at 30°C under reaction conditions which permitted polyadenylation, and the resultant labeled RNA was extracted and directly analyzed by gel electrophoresis under denaturing conditions. Titrating HeLa nuclear extracts with increasing amounts of IgG directed against purified hepatoma enzyme (Fig. 2, lanes 4 to 6) resulted in a proportional decrease in the polyadenylation

FIG. 2. Inhibition of adenovirus L3 mRNA polyadenylation *in vitro* by anti-poly(A) polymerase antibodies. HeLa nuclear extracts (8 μ l) were preincubated with various amounts of antihepatoma poly(A) polymerase IgG (27 μ g/ μ l) (lanes 3 to 6 [0, 0.5, 1, and 2 μ l, respectively]) as well as immune control IgG heated at 100°C (lanes 1 and 2) [1 and 2 μ l, respectively]) for 30 min on ice. 32 P-labeled precursor RNA, containing the adenovirus type 2 L3 polyadenylation site, and other required reaction components (see Materials and Methods) were then added to initiate the *in vitro* polyadenylation reaction. After a 2-h incubation at 30°C, reactions were terminated by the addition of a proteinase K stop buffer and phenol extracted. The reaction products were then analyzed on 8% polyacrylamide-7 M urea gels after autoradiography. PRE, Precursor RNA (267 nucleotides); POLY A⁺, polyadenylated RNA. Lane M, DNA size markers (32 P-labeled fragments from a *Hind*III digestion of bacteriophage ϕ X174); arrow corresponds to the 271- and 281-nucleotide fragments of this digestion.

activity relative to the control sample incubated in the absence of antibodies (lane 3). A gradual reduction in the appearance of the polyadenylated products accompanied by an increase in unprocessed precursor RNA was observed when increasing amounts of the antibody were added to the extracts. In contrast, inclusion of immune IgG heated at 100°C (lanes 1 and 2) at a concentration shown to inhibit polyadenylation completely (compare lanes 2 and 6), as well as IgG purified from preimmune rabbit sera (data not shown), did not inhibit polyadenylation activity.

Manganese-dependent polyadenylation activity is inhibited by anti-poly(A) polymerase antibodies. Studies with purified poly(A) polymerase isolated from a variety of sources have revealed that Mn^{2+} can stimulate the activity of a majority of these enzymes (for reviews, see references 6 and 15). Under identical reaction conditions, distinct reaction products were formed when precursor mRNA was incubated in the presence of equal concentrations of either Mg^{2+} or Mn^{2+} (Fig. 3A). A major difference was the size of the poly(A) tract generated under the two assay conditions. Inclusion of 0.5 mM Mg^{2+} resulted in production of a 200- to 250-nucleotide-long poly(A) tract, of the approximate length observed *in vivo*. In contrast, in the presence of 0.5 mM Mn^{2+} , poly(A) tracts ranging from 400 to 800 nucleotides long were added. To test whether the enzyme activated in the presence of Mn^{2+} differed from that functioning in the presence of Mg^{2+} , the effect of anti-poly(A) polymerase antibody on Mn^{2+} -dependent polyadenylation of precursor RNA was studied (Fig. 3B). Titrating these reactions with increasing amounts of antibodies resulted in a dose-dependent decrease in the amount of polyadenylated RNA products generated. A drastic reduction in poly(A) length occurred when relatively low concentrations of antibodies were added (Fig. 3B, lanes 4 and 5). A polyadenylated product approximately 50 nucleo-

FIG. 3. Inhibition of manganese-dependent polyadenylation by anti-poly(A) polymerase antibodies. (A) ^{32}P -labeled L3 pre-mRNA was incubated under standard polyadenylation conditions for 2 h at 30°C in the presence of either 0.5 mM magnesium chloride (Mg^{2+}) or 0.5 mM manganese chloride (Mn^{2+}). The purified RNA was analyzed by electrophoresis on an 8% polyacrylamide-7 M urea gel. (B) HeLa nuclear extracts (8 μl) were treated with increasing amounts (lanes 2 to 8 [0, 0.5, 1, 2, 3, 5, and 7 μl , respectively]) of anti-poly(A) polymerase IgG (36 $\mu\text{g}/\mu\text{l}$) or preimmune IgG (lane 1; 150 μg). The samples were incubated on ice for 30 min. Polyadenylation reactions were initiated by the addition of labeled RNA and other reaction components, including 0.5 mM manganese chloride, and allowed to incubate at 30°C for 2 h. The RNA was purified, followed by electrophoresis through an 8% polyacrylamide-7 M urea gel. Precursor (PRE) and polyadenylated forms of RNA (POLY A $^+$) are indicated. Lane M, DNA size markers (^{32}P -labeled fragments from a *Hind*III digestion of phage ϕX174); corresponds to the 603-nucleotide (top) and 234-nucleotide (bottom) fragments of this digestion. The other fragments (top to bottom) correspond to 1.35, 1.08, 0.872, 0.310, 0.281 and 0.271, 0.194, 0.118, and 0.072 kilobases.

tides longer than the precursor was the most persistent product as the concentration of antibody increased (lanes 6 and 7). A higher concentration of antibody was required to abolish the presence of this short poly(A) tract (compare lanes 5 to 7 with lane 8).

In the presence of Mn^{2+} and relatively high concentrations of antibody, the precursor appeared to be converted into smaller fragments of RNA that migrated slightly faster than did the unprocessed pre-mRNA. This effect was consistently observed and occurred only in the presence of Mn^{2+} and antibody; it was apparently not due to any intrinsic activity of the added antibiotics, since preimmune IgG (Fig. 3B, lane 1) or equal amounts of the immune IgG added to Mg^{2+} -containing reaction mixtures did not exhibit this profile. The apparent breakdown of the product cannot be attributed solely to Mn^{2+} in the reaction mixture, since degradation of the precursor was negligible in the absence of antibodies (lane 2).

Antibodies against poly(A) polymerase also inhibit specific endonucleolytic cleavage of pre-mRNA. Several lines of evidence indicate that polyadenylation is tightly coupled to cleavage at the poly(A) site both in vivo and in vitro. Cleaved products are not normally detected, presumably because the processed RNA is rapidly converted to the polyadenylated form. Addition of high concentrations of EDTA or replacement of ATP with an ATP analog such as cordycepin triphosphate (3'-dATP) in vitro is known to block polyadenylation activity selectively, with a retention of cleavage activity. Thus, chemical uncoupling of polyadenylation from cleavage has provided a successful means to assay for the specific endonucleolytic cleavage reaction.

To determine whether antibodies specifically directed against poly(A) polymerase would affect the formation of accurately cleaved RNA products, we examined the effects of these antibodies on cleavage activity. Reactions were carried out under polyadenylation conditions (ATP) or cleavage conditions (3'-dATP or EDTA) in the presence or absence of antibody. First, the effect of the antibodies was tested in reactions in which polyadenylation was uncoupled from cleavage by addition of EDTA. Reaction mixtures were probed with both immune and preimmune antibodies to evaluate the specificity of the antibody-mediated effect. Anti-poly(A) polymerase antibodies that inhibited polyadenylation also inhibited specific endonucleolytic cleavage of precursor RNA (Fig. 4, lane 9). This inhibitory effect was not seen when the antibodies were omitted (lane 8) or when an equal concentration of preimmune IgG was added (lane 10). Similar results were obtained with 3'-dATP under the same conditions (Fig. 4, compare lane 5 with lanes 4 and 6). Since it is possible to uncouple cleavage from polyadenylation by chemical means, antibodies specific for poly(A) polymerase had been expected to prevent polyadenylation but allow cleavage of the precursor mRNA. Inhibition of both cleavage (Fig. 4) and polyadenylation (Fig. 2) by the anti-poly(A) polymerase antibodies suggest that the poly(A) polymerase and endonucleolytic activities may be immunologically inseparable (see Discussion).

Poly(A) polymerase antibodies prevent cleavage when added at various stages of the reaction. To determine whether the antibodies could exert their inhibitory effect during ongoing 3' processing reactions, purified IgG was added at different time points of reaction under conditions which favored cleavage (Fig. 5). As expected, addition of anti-poly(A) polymerase antibodies to extracts 30 min before incubation at 30°C resulted in complete inhibition of cleavage activity, as judged by the lack of cleaved product (Fig. 5A, lane 1).

FIG. 4. Effect of poly(A) polymerase antibodies on endonucleolytic cleavage under conditions in which polyadenylation is uncoupled from cleavage by two different reagents. Reactions were performed at 30°C for 30 min under polyadenylation conditions (lanes 2 and 3) or conditions that inhibit polyadenylation but do not prevent cleavage (lanes 4 to 6 [1 mM 3'-dATP] or lanes 8 to 10 [2 mM EDTA]). As a control, lanes 1 and 7 contain RNA that was not incubated at 30°C. Nuclear extracts were pretreated (30 min on ice) with either 2.5 μ l of anti-poly(A) polymerase IgG (36 μ g/ μ l) (lanes 3, 5, and 9) or 100 μ g of preimmune IgG (lanes 6 and 10) or were incubated in the absence of antibody (lanes 2, 4, and 8). After RNA purification, samples were analyzed on 8% polyacrylamide-7 M urea gels. PRE, Unprocessed precursor RNA; POLY A⁺, polyadenylated RNA; cleaved, 5' RNA fragment generated upon accurate endonucleolytic cleavage at the L3 poly(A) site; M, DNA size markers, with the arrow corresponding to the 310-nucleotide (upper) and 234-nucleotide (lower) fragments of a *Hind*III digestion of phage ϕ X174.

Cleavage was also blocked when antibodies, substrate RNA, and other reaction components were added simultaneously to the nuclear extract and then incubated (lane 2). However, if the antibodies were introduced after the onset of the reaction, accurately cleaved products could be detected (Fig. 5A, lanes 3 to 5). Thus, there was a relationship between the time of antibody addition and the level of cleaved products generated. When reactions were allowed to proceed for longer periods before addition of antibody, a greater percentage of the precursor was converted to correctly cleaved products. In contrast, addition of an equal concentration of preimmune IgG at similar time points had no effect on cleavage activity (Fig. 5B).

Anti-poly(A) polymerase antibodies inhibit formation of polyadenylation-specific complexes. Recently, several laboratories have reported the formation of specific complexes when competent pre-mRNA substrates are incubated in HeLa nuclear extracts under conditions that allow 3'-end processing. The detection of such complexes has been accomplished both by sedimentation analysis on sucrose or glycerol gradients (30, 40) and by gel mobility shift assays on nondenaturing gels (14, 25, 38, 49, 50). The molecular events which lead to the formation of properly cleaved and polyad-

FIG. 5. Effect of anti-poly(A) polymerase antibodies added during various stages of the 3'-end processing reaction. Labeled RNA was incubated in nuclear extracts containing 1 mM cordycepin triphosphate (3'-dATP). In all cases, the samples were incubated at 30°C for 60 min. Anti-poly(A) polymerase IgG (90 μ g) (A) or preimmune IgG (100 μ g) (B) was added to the reactions at different time points. IgG was added 30 min before the start of the reaction (lane 1), at the start of the reaction (lane 2), or after the reaction had proceeded for 5, 15, or 30 min (lanes 3 to 5). The reaction was also carried out in the absence of IgG (lane 6). PRE, Unprocessed precursor RNA; cleaved, 5' RNA fragment generated upon accurate endonucleolytic cleavage at the L3 poly(A) site; M, DNA size markers, with the arrows corresponding to the 271- and 281-nucleotide (upper) and the 194-nucleotide (lower) fragments of a *Hind*III digestion of phage ϕ X174.

enylated RNA appear to occur within these RNA-protein complexes. It is therefore reasonable to assume that the poly(A) polymerase is likely to be present within the specific complexes during some stage of the polyadenylation event. To test this hypothesis, we investigated the effect of anti-poly(A) polymerase antibodies on the assembly of such complexes. For this purpose, equal portions of nuclear extracts from HeLa cells were incubated in the presence or absence of an inhibitory concentration of anti-poly(A) polymerase antibodies. ³²P-labeled adenovirus L3 pre-mRNA containing the polyadenylation signal was incubated in these extracts under polyadenylation reaction conditions over a 1-h time course (Fig. 6). to permit simultaneous analysis of both ribonucleoprotein (RNP) complex formation and RNA product generation, reactions were stopped at various time points; then half of the reaction was subjected to electrophoresis on nondenaturing polyacrylamide gels (Fig. 6A), and the other half was resolved on denaturing polyacrylamide-urea gels (Fig. 6B).

When polyadenylation reactions were allowed to proceed in the absence of antibodies (Fig. 6A, lanes 1 to 5), at least three distinct forms of RNP complexes, designated PA

FIG. 6. Effect of antihepatoma poly(A) polymerase antibodies on the formation of polyadenylation-specific complexes. HeLa nuclear extracts were preincubated for 30 min on ice in the absence ($-Ab$) or presence ($+Ab$) of an inhibitory concentration (total reaction volume, 98 $\mu\text{g}/25\ \mu\text{l}$) of IgG-purified hepatoma anti-poly(A) polymerase antibodies. Purified ^{32}P -labeled precursor L3 RNA was incubated in these nuclear extracts under polyadenylation reaction conditions, and the reactions were stopped at the specified time points. (A) 12.5 μl (half of the reaction mixture) was removed, and the reaction was terminated by the addition of heparin (final concentration, 5 mg/ml) on ice for 10 min. These samples were directly loaded onto and electrophoresed on nondenaturing 4% polyacrylamide gels. Positions of distinct complexes PA (preactive), A (active), P (polyadenylated product), and Ab.S (antibody specific) between substrate RNA and nuclear extracts are indicated. PRE, Unreacted precursor RNA. (B) The remaining 12.5- μl portion was digested in a proteinase K stop buffer to deproteinize the RNA and then purified, and the RNA reaction products were analyzed on 8% polyacrylamide-7 M urea gels. PRE, Unprocessed precursor RNA; POLY A $^{+}$, polyadenylated RNA; M, DNA size markers (^{32}P -labeled fragments from a *Hind*III digestion of phage ϕX174), with arrows corresponding to the 310-nucleotide (upper) and 271- and 281-nucleotide (lower) fragments of the digest.

(preactive), A (active), and P (polyadenylated), were produced. An initial complex (PA) was detected (lane 1) which appeared as a smear migrating slower than the free precursor (PRE). This complex probably reflected the rapid associa-

tion of HeLa heterogeneous nuclear RNP particles with the L3 RNA as it formed instantly even in the absence of incubation. Another complex (A), which displayed lower mobility than did complex PA, was assembled only after the

RNA was incubated at 30°C with the nuclear extract. This complex was first detected within 5 min (lane 2). A decrease (lane 3) followed by disappearance of both complexes PA and A and concomitant appearance of a new complex (P) was observed upon further incubation (lanes 4 and 5). Complex P migrated to a position below that of complex A and to a slightly higher, although partially overlapping, position at which complex PA migrated. At 60 min, P was the only observable complex.

Analysis of the RNA products generated at various stages of these reactions revealed that polyadenylated products accumulated over time (Fig. 6B, lanes 1 to 5). Furthermore, there was a positive correlation between the reduction in complex A, appearance of complex P, and detection of polyadenylated RNA products. Prior inclusion of an inhibitory concentration of the antibodies in the extract inhibited polyadenylation completely (Fig. 6B, lanes 6 to 9), as indicated by the lack of processing of the precursor RNA. Interestingly, when these same reaction samples were analyzed on nondenaturing gels, RNP complexes could still be observed (Fig. 6A, lanes 5 to 9). However, the antibody treatment affected the formation of complexes (Fig. 6A, compare lanes 5 to 9 with lanes 2 to 4). Thus, although the formation of complex PA was not prevented by antibodies, the disappearance of this complex over time was greatly diminished in the presence of antibodies. The most striking effect of the antibodies was manifested in the times of formation of complexes A and P. At early reaction stages (5 and 15 min), a complex (Ab.S [antibody specific]) that migrated in the native gel to a position below that of complex A was observed. The higher mobility of complex Ab.S relative to that of complex A suggested that Ab.S may lack a bound factor(s) present in complex A formed in the absence of antibodies. Later in the course of the reaction (30 and 60 min), the shift in RNA mobility corresponding to Ab.S was not detected. Antibodies completely prevented the formation of complex P, which is further consistent with the relationship between the formation of this complex and the generation of polyadenylated products.

Effect of anti-poly(A) polymerase antibodies on complex formation in reactions favoring only cleavage. Next, we examined the kinetics of complexes generated in both the presence and the absence of anti-poly(A) polymerase antibodies in reactions in which ATP was substituted by the ATP analog cordycepin (3'-dATP) (Fig. 7). Under these conditions, only the cleavage reaction could occur (Fig. 4 and 5). A temporal analysis of the RNA-protein complexes revealed that complex PA was formed in the absence of antibodies. Within 5 min, a decrease in PA concurrent with the appearance of complex A was observed (Fig. 7, lanes 1 to 5). Unlike the case with reactions performed in the presence of ATP, complexes PA and A remained stable over the 60-min time course in the presence of 3'-dATP. Furthermore, complex P did not form, which indicated its role exclusively in the polyadenylation reaction. When reactions containing 3'-dATP were first incubated with antibodies and analyzed for complex formation, complex PA could be assembled but formation of complex A was prevented (Fig. 7, lanes 6 to 9). As was the case with samples incubated in the presence of ATP, complex Ab.S could be detected in the presence of 3'-dATP. This complex assembled within 5 min in the presence of antibodies and was no longer detectable after 30 min.

FIG. 7. Effect of antihepatoma poly(A) polymerase antibodies on the formation of RNP complexes generated under conditions that uncouple polyadenylation and cleavage. 32 P-labeled precursor RNA and cofactors required for detecting cleaved but not polyadenylated RNA products (i.e., 1 mM 3'-dATP [see Materials and Methods]) were added to HeLa nuclear extracts that had been previously treated with (+Ab) or without (-Ab) an inhibitory concentration of IgG-purified antibodies (98 μ g per reaction). The cleavage reaction mixtures (total volume, 25 μ l) were incubated at 30°C for the specified times. (A) In half of the sample (12.5 μ l), the reaction was stopped by the addition of heparin at a final concentration of 5 mg/ml. These samples were then analyzed after electrophoretic separation on nondenaturing 4% polyacrylamide gels, followed by autoradiography. The left lane contains precursor RNA that had not been added to nuclear extracts. Positions of complexes PA (preactive), A (active), and Ab.S (antibody specific) are indicated.

DISCUSSION

The primary objective of this study was to establish the direct role of poly(A) polymerase in mRNA polyadenylation. Although the enzymatic, structural, and immunologic features of this enzyme have been investigated in great detail (for reviews, see references 6 and 15), the function of the enzyme in the 3'-end processing of mRNA and the mechanism by which it carries out this unique processing have not been deciphered. The enzyme from different sources, which consists of a single polypeptide, was usually characterized by its ability to add adenylate residues to the 3' terminus of a synthetic primer such as poly(A) or an RNA molecule (15). These studies have not revealed the specificity of the polyadenylation reaction as it occurs *in vivo* or in the cell-free systems containing the factors essential for polyadenylation. The lack of specificity of purified poly(A) polymerase has cast doubt on the physiological role of this enzyme. Attempts to fractionate nuclear extracts have yielded a fraction containing poly(A) polymerase which, in concert with some

other fraction(s), can yield correctly polyadenylated mRNA (22, 43). However, since the enzyme used in these studies is likely to contain other polypeptides, it is not certain whether the poly(A) polymerase characterized in earlier studies is the functional enzyme. The observation presented here that antibodies against purified poly(A) polymerase could inhibit polyadenylation of a specific pre-mRNA has provided direct proof for the role of this enzyme in the 3'-end processing of mRNAs.

Another key issue addressed in this investigation is the coupling of endonucleolytic cleavage with the polyadenylation reaction. Previous studies have suggested that cleavage and polyadenylation might be tightly coupled processes, with no identifiable lag phase occurring between the two events. First, mutations in the hexanucleotide sequence AAUAAA, the polyadenylation signal located 10 to 30 nucleotides upstream of the polyadenylation site of most mRNAs, affect both cleavage and polyadenylation (47). Second, cleaved products are not detected *in vivo* and are not normally observed *in vitro* except under specific reaction conditions. Thus, polyadenylation and cleavage reactions could be uncoupled *in vitro* only by treatment with either EDTA or certain ATP analogs such as 3'-dATP (Fig. 4 and 5; 28, 37). A rigid requirement for divalent metal ions and ATP for polyadenylation, but not for cleavage, could explain the selective inhibition of polyadenylation and persistence of cleavage in the presence of EDTA and 3'-dATP. Finally, a recent investigation by Takagaki and co-workers (43) has shown that fractionation of HeLa nuclear extracts can resolve the poly(A) polymerase-containing fraction from the cleavage activity. However, efficient and accurate cleavage of three different pre-mRNAs requires reconstitution with the fraction containing poly(A) polymerase.

The study presented here has shown that both cleavage and polyadenylation can be inhibited *in vitro* by anti-poly(A) polymerase antibodies. The simultaneous inhibition of both 3'-end processing reactions by a specific inhibitor of poly(A) polymerase provides concrete evidence for the coupling of these two reactions. This observation suggests that generation of the poly(A) site is dependent on poly(A) polymerase in such a way that blocking polyadenylation by anti-poly(A) polymerase antibodies can cause a concomitant inhibition of the cleavage reaction. Conceivably, a poly(A) polymerase-dependent cleavage activity provides a mechanism which ensures rapid addition of poly(A) to the newly generated terminus and thereby protects the message from destruction by nuclear exonucleases. It can be argued that the inhibition of both cleavage and polyadenylation by anti-poly(A) polymerase antibodies is due to contamination of these antibodies with anti-endonuclease antibodies. This possibility can be ruled out for the following reasons. First, poly(A) polymerase used for raising antibodies exhibited a single 48-kDa band upon SDS polyacrylamide gel electrophoresis (41). Second, the 48-kDa antigen was eluted from the gel before the rabbits were immunized. Third, the antisera exhibited a single 48-kDa band after Western blot analysis (41; Fig. 1). Fourth, purified poly(A) polymerase did not exhibit endonuclease activity in an *in vitro* assay (data not presented). In fact, poly(A) polymerase activity present in HeLa extracts can be separated from endonucleolytic activity by initial chromatography on a DEAE-Sepharose column (43). Since we used a similar protocol for the purification of poly(A) polymerase (41), the antigen used for immunization should also be devoid of the specific endonuclease. It is also possible that a separate endonucleolytic activity that can be expressed *in vivo* and under certain *in vitro* conditions is

inherent in the poly(A) polymerase molecule. However, the close association of polyadenylation and cleavage activities in a functional complex would adequately explain the inhibition of both activities by anti-poly(A) polymerase antibodies. Poly(A) polymerase may exist as a complex with other components or, alternatively, the cleavage-polyadenylation may be brought about by separate components which form such a complex only after pre-mRNA is added. Immune complex formation between poly(A) polymerase and the antibodies could prevent formation of the complex or interaction of the components to form the complex with the precursor RNA.

Recent studies have shown that complexes can be formed during polyadenylation. Thus, electrophoretic mobility shift analysis of reaction samples has revealed the formation of 3'-end processing-specific complexes with herpes simplex virus thymidine kinase (50), herpes simplex virus type 2 (25), adenovirus L3 (14, 38), and simian virus 40 late (49) pre-mRNA substrates. Similar complex formation has also been observed after glycerol (30) or sucrose density gradient (40) fractionation of the product formed with adenovirus L3 mRNA. These studies have not extensively explored the nature of the components that make up these complexes. Recent studies in our laboratory have demonstrated that newly synthesized poly(A) polymerase is associated with four polypeptides of molecular sizes 74, 35, 30, and 22 kDa (J. Hengst, I. Georgieff, H. Isom, and S. T. Jacob, *J. Biol. Chem.*, in press). In another study, we have shown that poly(A) polymerase is closely associated with U1 small nuclear RNA (32), which is consistent with the inhibition of polyadenylation reactions with human lupus sera containing anti-RNP antibodies (27). Although the role of small nuclear RNPs in the cleavage-polyadenylation reaction is still unknown, several studies have raised the possibility that a small nuclear RNP component is essential for the cleavage reaction (1, 10, 12, 27, 40). The data presented here provide substantial proof for the existence of a functional complex that could play a key role in regulating the cleavage and polyadenylation reactions in a concurrent manner.

It is noteworthy that the divalent metal ions Mg^{2+} and Mn^{2+} exhibit different effects on the polyadenylation reaction. Mn^{2+} facilitates elongation of the poly(A) chain (Fig. 3A), which is consistent with our earlier finding that the free (unbound to chromatin) population of poly(A) polymerase activated in the presence of Mn^{2+} directs elongation of the poly(A) tract (33). Although the bound enzyme has been previously designated as chromatin bound, it is probably associated with the complex involved in the cleavage-polyadenylation (43). Therefore, it is possible that the Mg^{2+} -activated poly(A) polymerase is involved in the initial complex formation and polyadenylation, whereas the free population of poly(A) polymerase catalyzes the elongation of the initial poly(A) tract. The amount of antibodies required for achieving inhibition of polyadenylation in the presence of Mn^{2+} was threefold higher (data not shown). A potential alteration in the configuration of the complex in the presence of Mn^{2+} may have rendered poly(A) polymerase less readily accessible to the antibodies. The complete inhibition of Mn^{2+} - or Mg^{2+} -dependent polyadenylation by the antibodies negates the existence of two distinct species of poly(A) polymerases, each activated by a different divalent metal ion. Interestingly, earlier studies (35) in our laboratory, dealing with the characterization of purified poly(A) polymerases, reached similar conclusions. Another difference between Mg^{2+} - and Mn^{2+} -dependent polyadenylation has been in regard to the stability of the precursor after treat-

ment with anti-poly(A) polymerase antibodies. Unlike Mg^{2+} , Mn^{2+} appears to cause degradation of the RNA substrate after inhibition of polyadenylation by the antibodies (compare Fig. 2 with Fig. 3B). It should be emphasized that such breakdown of pre-mRNA occurs only when both Mn^{2+} and antibodies are present. Under these conditions, the mRNA precursor might not be protected by poly(A) polymerase or its associated components, which might result in substrate breakdown. This process might, at least, in part, be brought about by a specific Mn^{2+} -activated 3',5'-exonuclease, since the 5' termini are stabilized by capping during the reaction.

A striking observation has been the correlation between inhibition of polyadenylation complex formation and of poly(A) addition to cleaved pre-mRNA in the presence of anti-poly(A) polymerase antibodies (Fig. 6). Altogether, three complexes, designated PA (preactive), A (active), and P (polyadenylated), were produced with increasing time of incubation of pre-mRNA with the extract. These complexes are similar to those reported by other investigators (14, 25, 38, 49, 50). Complex PA was observed in the absence of polyadenylation, whereas complex A was detected under all conditions favoring polyadenylation. It is conceivable that complex PA does not contain poly(A) polymerase and that conversion of PA to A is contingent on association with poly(A) polymerase. A significant observation is the decrease in the level of complex A upon polyadenylation, with no significant alteration of this complex during cleavage. It is evident that complex A is essential for cleavage and polyadenylation, whereas conversion of A to P results during polyadenylation. The requirement of complex A for 3'-end processing is consistent with similar conclusions reached by indirect means (14, 25, 38, 49, 50).

The appearance of complex Ab.S (antibody specific), which formed in the presence of an inhibitory concentration of anti-poly(A) polymerase antibodies and concurrent with the inhibition of complexes A and P, deserves comment. The kinetics of Ab.S formation were similar to those of complex A formation. As was observed with complex A, complex Ab.S dissociated with time and was not detectable at later stages of the reaction. There are two potential models which could explain this process. In one model, poly(A) polymerase is presumed to be part of complex Ab.S. The antibodies may not prevent binding of the enzyme to the complex but inhibit its function, probably as a result of interaction with the ATP-binding site. The inability of the enzyme to function could result in release of the nonpolyadenylated RNA. Binding of the antibodies to the complex could induce a conformational change within the complex, which might be responsible for the slightly faster electrophoretic mobility of Ab.S relative to A. Such alterations in the structure of the complex could also lead to release of the precursor RNA. Alternatively, poly(A) polymerase may not be a component of complex Ab.S. Binding of the antibodies to the complex may prevent interaction of the enzyme with pre-mRNA or perhaps prevent its association with other nuclear components. The relatively faster migration of Ab.S might result from the absence of a factor which may indeed be poly(A) polymerase. We are now testing which of the models is valid. Complex Ab.S may actually represent a normal intermediate in the 3'-end processing reaction and may contain the components required for the initial stages of the reaction.

Another significant issue emerging from these studies concerns the nature of factors that constitute the complexes. Hashimoto and Steitz (12) have demonstrated that a factor with the properties of an sm small nuclear RNP associates

with the AAUAAA sequence upstream of the poly(A) site in pre-mRNA. UV cross-linking studies have identified a 64-kDa polypeptide that can interact specifically with the hexanucleotide sequence in precursor RNAs containing the poly(A) sites (45). This factor appears to be a component of the cleavage machinery. Humphrey and colleagues (14) have reported that a 67-nucleotide region encompassing the hexanucleotide and G+U-rich region of L3 mRNA can be protected by a factor associated with complex A after digestion with RNase T1. Zarkower and Wickens (48) have shown that a factor forms a very stable precleavage complex with the AAUAAA sequence. These investigators have postulated that the stability of the factor-hexanucleotide complex is due to a close and specific interaction between poly(A) polymerase and the 3' end of the RNA substrate. We are currently testing which of the complexes contain poly(A) polymerase. Direct Western blot analysis of the complexes has met with technical difficulties. Further analysis of the complexes using alternate approaches with the antibodies is in progress. This study will ultimately provide important information concerning the various stages of the 3'-end processing of mRNA in which poly(A) polymerase participates.

While this paper was under review, Christofori and Keller (3) reconstituted purified fractions containing poly(A) polymerase, a cleavage factor, and a cleavage and polyadenylation factor and achieved accurate cleavage and polyadenylation of pre-mRNA containing the adenovirus L3 polyadenylation site. These authors concluded that poly(A) polymerase activity of the fraction containing this enzyme is not the only function of this fraction and that it must participate directly or indirectly in the cleavage reaction. Thus, two independent studies using two entirely different approaches have demonstrated a dual role for poly(A) polymerase. Furthermore, our data strongly suggest that the previously characterized poly(A) polymerase (15, 16, 41) is indeed responsible for this key molecular event.

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