To the Graduate Council:

I am submitting herewith a thesis written by Marilyn Nelson Maupin entitled "Selection and Analysis of a Yeast Genomic DNA Clone that Encodes a Peptide Sequence Which Affinity-Purifies an Antibody for Poly(A) Polymerase of Yeast." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

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Selection and Analysis of a Yeast Genomic DNA Clone That Encodes a Peptide Sequence Which Affinity-Purifies an Antibody for Poly(A) Polymerase of Yeast

A Thesis

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Degree

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ABSTRACT

Poly(A) polymerases catalyze the addition of poly(A) tracts to the 3′ termini of mRNA and hnRNA. The identification of genes encoding the poly(A) polymerases and associated proteins will aid in understanding the mechanisms involved in this important RNA processing event and also the function of the poly(A) tails. Yeast is a unique eukaryote which offers an excellent system for this type of investigation.

A partially purified poly(A) polymerase with an estimated molecular weight of 66,000 was prepared in our laboratory and made possible the preparation of a polyclonal antibody from a New Zealand rabbit. The antibody reacted with a 66 kDa protein band on Western blots and also immunoprecipitated poly(A) polymerase activity.

A yeast genomic DNA library constructed in λgt11 was screened with the antibody, and two positive clones were selected that encoded a polypeptide which affinity-purified the polyclonal antibody. In Northern analysis, the selected clone hybridized a 1.3 kb mRNA and a 1.8 kb mRNA. The 1.8 kb mRNA is of a size that could encode a 66 kDa protein.

A 2 kb *Eco*RI fragment of the positive clone was ligated into the yeast centromere plasmid, pRS316, which was used to transform *E. coli* and yeast by electroporation. The transformed yeast showed no overexpression of poly(A) polymerase activity or overproduction of the 1.8 mRNA, so the 2 kb *Eco*RI fragment was ligated into a high-copy number plasmid, YEP24. This plasmid was used to transform *E. coli* and yeast in order to test for overexpression of poly(A) polymerase activity and overproduction of the 1.8 kb mRNA. Again, no overproduction of either the poly(A) polymerase nor the 1.8 kb mRNA was seen, and it seemed probable that only a portion of the poly(A) polymerase gene was present.

In order to obtain an intact poly(A) polymerase gene, a yeast genomic library containing larger DNA fragments in the high-copy number vector, YEP24, was screened by colony hybridization using the 2 kb *Eco*RI fragment as a probe. Two positive clones with inserts of 10-13 kb were obtained and both showed overproduction of a 1.3 kb mRNA and a 1.8 kb mRNA, but neither clones gave any significant overexpression of poly(A) polymerase activity.

Restriction enzyme mapping was carried out with the YEP24 clones, and the map was compared to a restriction map of a gene coding for a 63 kDa poly(A) polymerase cloned by Lingner et al. The two maps showed no similarity.

Additional Western blot analysis was carried out to see if the affinity-purified antibody reacted with other protein bands in crude extracts and with DE-23 cellulose chromatography fractions of yeast containing the control plasmid YEP24 and the YEP24 clone. Two bands at approximately 66kDa were detected but neither was overexpressed by the YEP24 clone. There was overexpression of a 54kDa protein band and it is probable that this is the protein which is encoded by the clone described in this study.

The affinity-purified antibody may be useful in analyzing whether yeast contains several poly(A) polymerases. Further sequencing of the clone should be carried out to determine if it is homologous to clones of poly(A) polymerase of higher eukaryotes.

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CHAPTER I

INTRODUCTION

Edmonds and Abrams in 1960 described an enzyme from calf thymus nuclei which catalyzed the synthesis of poly(A) from ATP and which required RNA as a primer (1). The discovery of poly(A) polymerase took on a new significance when poly(A) tracts were found at the 3′ termini of mRNA and hnRNA in eukaryotes (2-5). Poly(A) tracts are added after transcription (6) and poly(A) polymerases catalyze the reaction (7-10).

Many studies have described the purification and characterization of poly(A) polymerases (PAPs) from different organisms (1,11-15,18,20,43). All of the poly(A)polymerases catalyze the reaction shown below (20).

$$\text{Polyribonucleotide} + n\text{ATP} \xrightarrow{Mg^{2+} \text{ or } Mn^{2+}} \text{Polyribonucleotide(A)n} + n\text{PPi}.$$

In yeast, a predominant nuclear poly(A) polymerase has been partially purified (21,22) and, while this work was in progress, Lingner et. al. (23) reported a 22,000 fold purification to homogeneity of a 63 kDa poly(A) polymerase from whole cell extracts of *Saccharomyces cerevisiae* with a 22% yield. Haff and Keller (22) reported that 75% of the total PAP activity of yeast is poly(A) polymerase I. Two other poly(A) polymerase activities can be separated by DEAE-cellulose chromatography and account for 20% and 5% of the total activity found in crude extracts (21,22). Poly(A) polymerase II requires

a primer with an oligo(A) segment at the 3′ terminus while poly(A) polymerase I requires no oligo(A) segment at the 3′ terminus of its RNA primer. This suggests that poly(A) polymerase I can initiate short oligo(A) sequences on RNA of mixed composition and then the poly(A) polymerase II utilizes the product of poly(A) polymerase I and elongates it further (21). Poly(A) polymerase I was found to elute from DEAE-cellulose columns at 0.07 M $(NH_4)_2SO_4$ and PAPII at 0.20 M $(NH_4)_2SO_4$ (22). There is also evidence for more than one poly(A) polymerase from calf thymus (16,43), rat liver (14,15), *E. coli* (11), and HeLa cells (13).

The specific role of the 3′ poly(A) tail on nuclear and cytoplasmic mRNAs is not fully understood. The polyadenylation state of an mRNA determines the efficiency of its translation (24,25). Wickens and Stephenson found that only those RNA molecules which are polyadenylated are found in the cytoplasm. This implies poly(A) tracts may be involved in nuclear to cytoplasm transport and/or cytoplasmic stability (26). Work by Drummond et. al. indicates that polyadenylated synthetic mRNAs show an increase in stability and translation efficiency (27). Polyadenylation also acts to signal the RNA polymerase II to terminate transcription (28), as described below.

Polyadenylation in eukaryotes is coupled to a cleavage of the primary transcript at the 3′ end of the mRNA and the polyadenylation of the newly formed end (29-32). The poly(A) signals in mammals are now known to contain two elements, an AAUAAA sequence 20-30 nucleotides upstream and a diffuse GU-rich sequence immediately after the 3′ end of the mRNA. Other elements upstream of the polyadenylation site may influence its efficiency (33,34). In *S. cerevisiae* instead of the AAUAAA signal, a diffuse AU-rich element is often found. Studies of the *CYC1* gene and mRNA suggest that polyadenylation signals are also termination signals and that the two processes are coupled (35,36). Butler et. al. (37) have described an in vitro polyadenylation system using a number of mRNA substrates and showed that mRNA 3′ end formation in yeast involves a cleavage reaction with subsequent polyadenylation as described for higher eukaryotic (29-32).

Identification of genes encoding poly(A) polymerases and associated proteins will help further understanding of the mechanisms involved in the formation of the poly(A) termini of mRNA and the function of the poly(A) tails. Yeast offers an excellent system for this type of investigation, making possible the utilization of both biochemical and genetic techniques. The availability of a partially-purified poly(A) polymerase fraction in our laboratory made possible the preparation of a polyclonal antibody and initial studies to clone the gene or genes for yeast poly(A) polymerases. These studies constitute this thesis and are described below.

CHAPTER II

MATERIALS AND METHODS

Preparation of Antibody

A poly(A) polymerase fraction of *Saccharomyces cerevisiae* was purified 1000-fold according to a procedure developed in our laboratory and described briefly below (p. 10). As shown under Results, the enzyme fraction shows a major protein band of 66 kDa on SDS-polyacrylamide gel electrophoresis. The 66 kDa band parallels poly(A) polymerase activity. The 66 kDa band was excised from gels and used as an antigen for the immunization of a young female New Zealand rabbit as described by Eberle & Courtney (38). The rabbit was injected subcutaneously, and the serum was collected by ear-bleeding 2 weeks after the fifth immunization. As described under Results, polyclonal antibody was produced which reacted with the 66 kDa band on Western blots carried out by the method of Burnette (39). Poly(A) polymerase activity was also immunoprecipitated by the antibody using the method of Kennel (40).

Immunoscreening λgt11 Library

The bacteriophage expression vector λgt11 (*lac5 nin5 c*T857 *S*100) containing a library of yeast genomic DNA-fragments inserted in *Eco*R1 linker sites was obtained from Dr. Michael Snyder, Yale University, and screened using the poly(A) polymerase polyclonal antibody according to the procedure of Snyder et. al. (41). Blocking of nonspecific protein binding sites was carried out with TBS (150 mM NaCl, 50 mM Tris-HCl, pH 8.1) + 5% Blotto. Immunopositive clones were detected by using alkaline phosphatase (AP) conjugated secondary antibody and AP color development reagents BCIP and NBT (BioRad) (42).

Plaque purification of recombinant λgt11 was carried out by removing an agar plug containing the plaque, incubating it in 1 ml of SM (Appendix), and replating the phage. The plating and screening procedure was repeated until all the plaques on the plate produced a positive signal.

Affinity Purification of Polyclonal Antibody Using λgt11 Clones

The λgt11 clones were analyzed according to the procedure of Zassenhaus et. al. (43) to see if the peptide sequence which they encoded affinity purified the polyclonal antibody. The positive λgt11 recombinants were plated at 5×10^4 pfu/ml (2 plates per recombinant) and the immunoscreening methods were carried out up to the point of antibody probing. The filters were then incubated with the antibody solution overnight using 10 ml of a 1:50 dilution per 2 filters. The filters were washed in 5% Blotto – TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5). The antibody was then eluted from the filters using 0.1 M glycine-HCl (pH 2.9), 0.1 M NaCl, 1% BSA w/v, 0.1% NP-40 v/v, and 10% ethylene glycol v/v. The eluted antibody was quickly neutralized with 1 M Tris-OH, and used as described under Results.

Ligation of Restriction Enzyme Fragments into Plasmid DNA and Transformation of *E. coli* and *S. cerevisiae*

Preparation of DNA

The λgt11 recombinant bacteriophage containing the clones that affinity-purified the antibody was mixed at 10^5 pfu with 0.1 ml *E. coli* Y1090 (41), and after a 20 min incubation at 37°C was poured onto a 100 mm LB plate (Appendix). The plate was incubated without inversion for 9 h at 37°C. After confluent lysis, 5 ml of SM was added to the plate and incubation was continued overnight at 4°C. The SM wash was collected (4 plates for a total of 20 ml), 0.3 ml chloroform was added, and after brief vortexing the mixtures were centrifuged at 4000 x g for 10 min at 4°C. The bacteriophage was recovered and purified by the Yamamoto procedure (33). The cesium chloride gradient was

centrifuged in an SW 50.1 rotor at 35,000 rpm for 23 h at 4°. The phage band was removed from the gradient and dialyzed against 2 liters of 10 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 10 mM $MgCl_2$ for 3.5 h, changing to fresh dialysis buffer after the first hour. The bacteriophage band was transferred to a sterile corex conical graduated tube and the following were added: EDTA to 2mM, proteinase K to 0.5%, and sodium dodecyl sulfate (SDS) to 0.5%. After vortexing several times the bacteriophage were incubated for 1 h at 65°C. An equal volume of STE (Appendix) saturated phenol was added and the tube was inverted several times to mix. After centrifugation at 1600 x g for 5 min the aqueous phase was transferred to a clean tube and extracted an additional two times with 50:50 phenol-chloroform. The aqueous phase was dialyzed against three 200-fold volumes of TE (1 mM EDTA, 10 mM Tris, HCl, pH 8.0) at 4°C. The DNA was transferred to an Eppendorf tube (1.5 ml) and stored on ice.

Preparation of *Eco*RI Fragment

*Eco*RI restriction endonuclease was purchased from Bethesda Research Laboratories, and the λgt11 phage DNA was completely digested with *Eco*RI using the methods and buffers provided by the supplier. The digested DNA was electrophoresed in 1% BRL low-melting-temperature agarose gels in 1 × TBE (Appendix). The 2 kb *Eco*RI fragment contained in the clones was cut out from the gel and incubated in 1 ml STE for 15 min at room temperature. The STE was discarded and the gel slice was incubated at 70°C for 10 min to melt the agarose. It was then incubated at 37°C for 5 min and EDTA was added to 5 mM and NaCl to 50 mM. Agarase (Calbiochem) was added to 100 units/ml and incubation was continued overnight at 37°C. An equal volume of PCA (50% phenol, 48% chloroform, and 2% isoamyl alcohol) was added to the agarose digest, and it was vortexed briefly. After spinning at 13,000 rpm for 5 min, the aqueous layer was removed and Na acetate was added to 0.15 M. The DNA was precipitated with two volumes of 100% ethanol, spun, and further washed with 95% ethanol. The pellet was dried in a vacuum desiccator and then dissolved in 10 μl of TE.

Ligation of 2 kb Insert into Yeast Centromere Plasmid pRS316 and Transformation of *E. coli*

T4 DNA ligase was purchased from BRL and ligation of the 2 kb *Eco*RI fragment into the pRS316 polylinker (45) was performed using the methods and buffer supplied by the vendor. pRS316 was obtained from Dr. Frank Larimer (Oak Ridge National Laboratory, Oak Ridge, Tennessee).

Transformation of *E. coli* XL1-Blue (46) with pRS316 containing the 2 kb DNA fragment was carried out by high voltage electroporation (47). The XL1-Blue and the Gene Pulser apparatus (Bio-Rad Laboratories) was provided by Dr. Larimer. A Gene Pulser cuvette (BioRad) (0.2 cm) was used and exponential decay pulses were generated by setting the apparatus at maximum voltage (2.5 kV) with the pulse controller capacitance at 25 μF and resistance at 200 Ω. The transformed cells were plated on LB agar containing 50 μg/ml ampicillin, 100 mM IPTG, and 2% X-Gal. White colonies were selected and the bacteria were grown in LB media + 50 μg/ml ampicillin.

Transformation of *S. cerevisiae* YNN27 with pRS316

DNA minipreps of XL1-Blue transformants were prepared by the alkaline lysis method (44). Transformation of *S. cerevisiae* strain YNN27 (α ura3-52 trpl-289), a gift of R. Davis, Stanford University, was carried out as described by Simon and McEntee (48). An electrode gap of 0.4 cm was used with voltage set at 1000 and capacitance set at 25 μF to give a field strength of 2500 V/cm. Transformants were plated by spreading aliquots on SC plates minus uracil (Appendix). The positive YNN27 transformants were grown in media containing 6.7 g/liter yeast nitrogen base, 0.5% casamino acids, 5% dextrose, and 20 μg/ml tryptophan. The YNN27 control was grown in the same media plus 20 μg/ml uracil.

Ligation of 2 kb Insert into High Expression Vector YEP24 and Transformation of *E. Coli* and YNN27

The restriction endonucleases *Sal*I and *Sma*I were purchased from BRL and the YEP24(49) was obtained from Dr. Larimer. The ligation protocol described above was used for the insertion of the 2kb *Sal*I/*Sma*I restriction fragment of the pRS316 clone into the *Sal*I/*Sma*I restriction sites of the high-copy number shuttle plasmid YEP24.

Transformation of *E. coli* XL1-Blue and yeast strain YNN27 with the plasmid containing the 2 kb fragment was carried out by electroporation as described above.

Testing XL1-Blue and YNN27 Transformants for Overexpression of Poly(A) Polymerase Activity

XL1-Blue

The *E. coli* XL1-Blue transformants were grown in LB + 50 μg/ml ampicillin at 37°C to an A_{650} of 3.0. The cultures were centrifuged in a Sorvall GSA rotor at 10,000 rpm for 10 min, washed with poly(A) polymerase (PAP) buffer (Appendix), and suspended in four times the volume of PAP buffer. The cells were passed through a French pressure cell (American Instrument Co.) three times at 4000 PSI and centrifuged two times in the Sorvall SS-34 rotor at 10,000 rpm for 10 min. The supernatant was spun in a Spinco Ti50 rotor at 45,000 rpm for 2 h, and the supernatant was assayed for poly(A) polymerase activity (Appendix).

YNN27 Extracts

The YNN27 transformants were grown at 30°C to an A_{650} of 8.0. The cells were harvested by centrifugation at 8000 rpm for 10 min in a Sorvall GSA rotor at 4°C, and washed once with ice cold sterile H_2O and once with PAP buffer. The cells were suspended in 8 ml PAP buffer per 5 g of cells and passed through the French pressure cell two times at 20,000 PSI. The extract was centrifuged at

10,000 rpm for 10 min in a Sorvall SS-34 rotor at 4°C, and the supernatant was centrifuged for 30 min at 15,000 rpm. This supernatant was further centrifuged at 45,000 rpm for 3 h at 4°C in a Spinco Ti50 rotor, and the supernatant was removed to assay for poly(A) polymerase activity.

RNA Isolation and Northern Analyses

RNA Isolation

Yeast or *E. coli* cells (20 ml culture in YPD or LB media, respectively) were grown to early log phase and harvested by centrifuging 10 min at 10,000 rpm in a Sorvall SS-34 rotor. The cells were suspended in 0.5 ml of 0.05 M Na acetate, pH 5.3, 0.01 M EDTA, 1% SDS. The cells were transferred to an Eppendorf tube and 0.55 ml of phenol (saturated with 0.05 M Na acetate, pH 5.3, 0.01 M EDTA) was added. The suspensions were held in a 68°C waterbath with frequent vortexing for 15 min. They were then cooled in liquid nitrogen until phenol crystals appeared, and spun at 13,000 rpm for 6 min in an Eppendorf centrifuge. A 0.45 ml aliquot of the aqueous layer was removed, and the phenol layer was extracted with 0.15 ml of 0.05 M Na acetate (pH 5.3), 0.01 M EDTA, 1% SDS as described and the aqueous layer combined with the first. Phenol extraction of the aqueous layer was repeated at 68°C for 6 min, vortexing once every minute. The aqueous layer (0.5 ml) was then extracted with 0.5 ml of 50:50 phenol (pH 5.3)/chloroform, vortexed several times and shaken for 10 min at 22°C. After spinning, 0.4 ml of the aqueous layer was transferred to a new tube and 1 ml of 100% ethanol was added to precipitate the RNA. After 60 min at -60°C, the RNA was collected by centrifugation. The RNA pellet was washed with 95% ethanol and dissolved in about 40 μl of 10 mM Tris, 7.5, 1 mM EDTA, 0.2% SDS.

Probes for Northern Analysis

The probes for the Northern analysis were suitable DNA fragments obtained by restriction enzyme digestion of the pRS316 cloned DNA. The probes were labeled using a nick translation kit (BRL) and the recommended procedures.

Northern Analysis

RNA analysis was carried out by Northern blotting as described by Sambrook, et al. (46). Nytran (Schleicher and Schuell) was used for RNA transfer. Hybridization was carried out in heat sealable pouches (Kopak Corp.).

YEP24 Colony Hybridization Screening and Transformation of Yeast

The YEP24 yeast genomic library was a gift of D. Botstein (Stanford University). The 2 kb *Eco*RI fragment of the positive λgt11 recombinants, subcloned into the *Eco*RI site of plasmid pRS316, was the source of the 2 kb *Eco*RI fragment used as a hybridization probe. The YEP24 colony hybridization screening was carried out as described by Sambrook et al. (46) using a DNA probe labeled with a nick translation kit (BRL) in the presence of [α^{32}P]dCTP obtained from Amersham. The positive YEP24 clones were analyzed by restriction enzyme analysis and used to transform YNN27 as described above.

Immunoprecipitation & Immunoblot Analysis

Column Chromatography of Yeast Strain YNN27 Extracts for Analysis of Antibody Reactivity

Yeast crude extracts were prepared as described under YNN27 Extracts (p. 8). Extracts were applied to a DE-23 cellulose column, and poly(A)polymerase activity was recovered by washing with DE-23 buffer (0.02 M Tris [pH 8.0], 0.5 mM EDTA, 10% glycerol, 0.1 mM DTT) + 0.1 M $(NH_4)_2SO_4$. Further purification was achieved by phosphocellulose column chromatography, eluting with a linear gradient of 0.07 M to 0.35 M $(NH_4)_2SO_4$ in DE-23 buffer. Peak fractions of the phosphocellulose

column were applied to a heparin agarose column and eluted with a linear gradient of 0.05 M to 0.6 M $(NH_4)_2SO_4$ in DE-23 buffer. Immunoprecipitation with peak fractions of the phosphocellulose and heparin agarose columns was carried out as described in the Appendix. Western blot analysis was done by the procedure of Burnette (39).

DEAE-Cellulose Chromatography of Yeast YNN27 and Clone-Transformed YNN27 Extracts for Analysis of Affinity-Purified Antibody Reactivity

Yeast extracts were prepared as described under YNN27 Extracts using PAP buffer and also with PAP Buffer + .5 M KCl. A high salt wash of the 45,000 rpm pellet was carried out with the low salt extracts by homogenization of the pellets in High Salt Wash Buffer (Appendix), stirring 1 h, and centrifugation for 3 h at 45,000 rpm. The supernatant was dialyzed overnight against 1 liter of DE-23 buffer, changing once. The dialyzed high salt wash was applied to a DE-23 cellulose column, eluted with DE-23 buffer + 0.1 M $(NH_4)_2SO_4$, and then eluted with DE-23 buffer + 0.3 M $(NH_4)_2SO_4$. Fractions were analyzed by Western blotting using 8% polyacrylamide gels (39). Affinity-purified antibody was used as a probe and antigen-antibody complexes were detected using AP conjugated second antibody as previously described.

Restriction Enzyme Mapping

Restriction enzyme digests on DNA isolated by the alkaline lysis method (44) were carried out using the protocol and buffers provided by the vendor. The digests were electrophoresed in 1% agarose gels in 1XTBE and stained with ethidium bromide.

CHAPTER III

RESULTS

Preparation of Rabbit Polyclonal Antibody for Poly(A) Polymerase

A poly(A) polymerase fraction was purified from *S. cerevisiae* S288C according to a procedure developed in our laboratory, described briefly under Materials and Methods (p. 10). The poly(A) polymerase activity was the major such activity in our extracts, and it seemed probable that it is the poly(A) polymerase I described by Haff and Keller (21,22). Fig. 1 shows a denaturing polyacrylamide gel of protein fractions, a crude extract and several of the most purified heparose agarose column fractions. Heparin agarose fraction 21 shows a band at 66 kDa, which paralleled the poly(A) polymerase activity through several chromatography steps. The 66 kDa gel bands of heparin agarose fractions 19-22 were used as an antigen. As the gel in Fig. 1 shows, a major contaminating protein band of about 64 kDa was found in the later heparin agarose fractions (24,25), and the 66 kDa gel bands injected into the rabbit were slightly contaminated with this band.

A New Zealand white rabbit was immunized with 10 μg of the 66 kDa band (heparin agarose fractions 19-22) every two weeks over a ten-week period as described under Materials and Methods and immune serum was collected. Fig. 2A shows a repeat of an SDS-polyacrylamide gel of a heparin agarose fraction (Gel 1) along with the gel (Gel 2) of Fig. 1 showing the heparin agarose fraction 21 lanes. The protein bands of Gel 2 were transferred to nitrocellulose for Western blotting. The Western blot analysis (Fig. 2B) showed that the immune serum of the rabbit reacted with the 66 kDa poly(A) polymerase band in heparin agarose fraction 21. Fig. 3 shows that the immune serum also immuno-precipitated the poly(A) polymerase activity of partially-purified fractions. About 80% of the poly(A) polymerase activity was removed from the supernatant solutions when 5 μl of immune

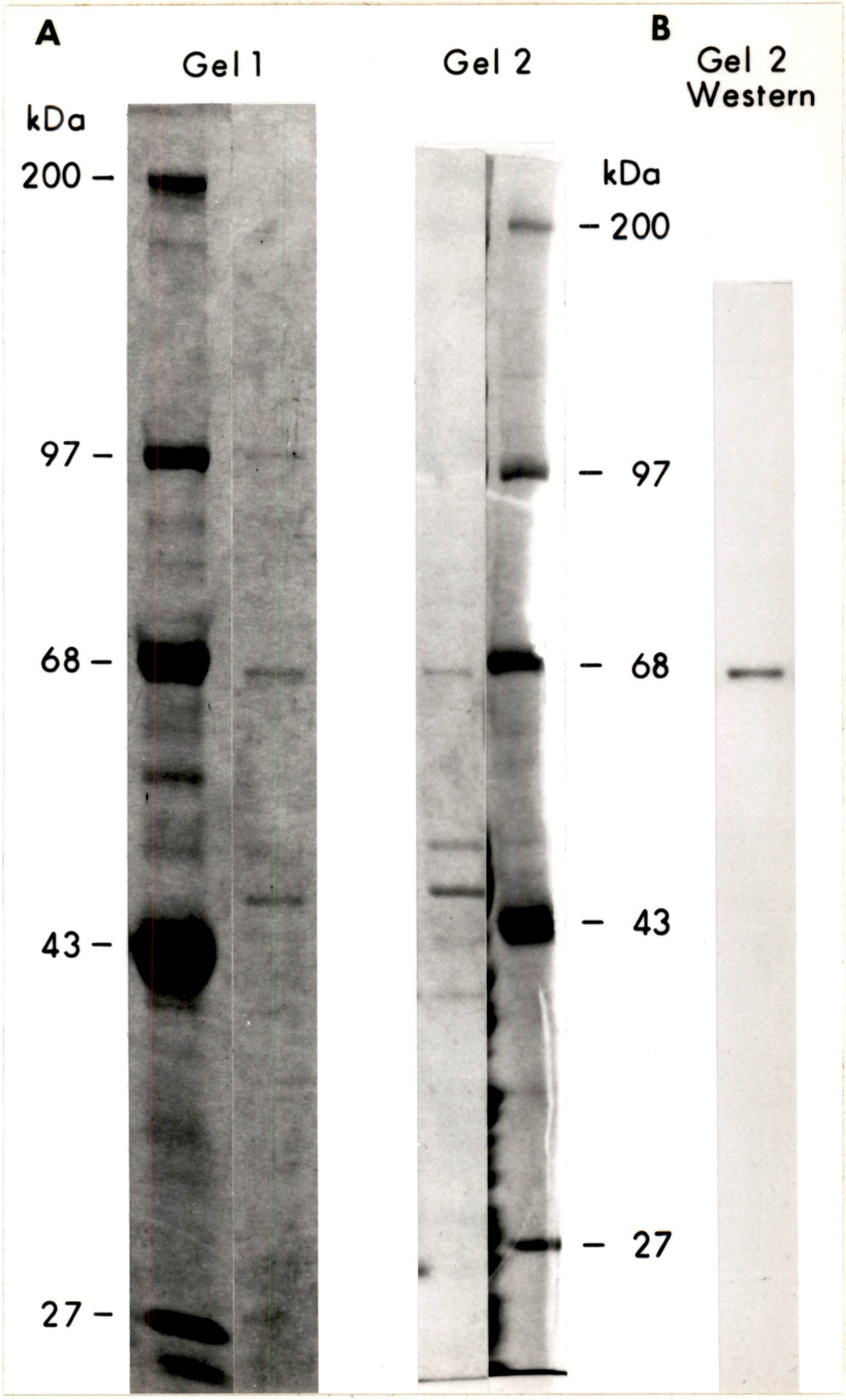
FIGURE 1. SDS-Polyacrylamide Gel Electrophoresis of Partially-Purified Poly(A) Polymerase Fractions of *S. cerevisiae*. Heparin agarose poly(A) polymerase fractions of *S. cerevisiae* were purified as described under Materials and Methods and loaded onto an 8% SDS-polyacrylamide gel (prepared and run as described by Laemmli) (56). Left to right, the lanes contain 25 μg heparin agarose fraction 25, 18 μg heparin agarose fraction 24, 8 μg heparin agarose fraction 21, 500 μg of crude extract, and molecular weight standard, with sizes in kDa shown on the right.

HEPARIN AGAROSE FRACTIONS
25
24
21
CRUDE EXTRACT
PROTEIN MARKERS
kDa
200
97
68
43
27

FIGURE 2. Western Blot Analysis of Partially-Purified *S. cerevisiae* Poly(A) Polymerase.

(A) Gel 1 is a second SDS-Polyacrylamide gel showing the 66 kDa protein band. Left to right, molecular weight standards with sizes in kDa shown to the left, 8 μg of heparin agarose fraction 21. Gel 2 is the SDS-Polyacrylamide gel used for Fig. 1. Left to right, 8 μg of heparin agarose fraction 21, molecular weight standards with sizes in kDa shown at the right.

(B) Western blot of heparin agarose fraction 21 (8 μg) hybridized with rabbit polyclonal antibody, according to the procedure of Burnette (39).


A
Gel 1
Gel 2
B
Gel 2
Western
kDa
200 –
97 –
68 –
43 –
27 –
kDa
– 200
– 97
– 68
– 43
– 27

Antibody used	% Activity remaining in supernatant		% Activity in pellet	
	phosphocellulose fraction 20	heparin agarose fraction 17	phosphocellulose	heparin agarose
Preimmune Serum				
2 μl	100	100	1.2	0
5 μl	100	100	6.0	0
Poly(A) polymerase immune serum				
2 μl	46	60	7	11
5 μl	15	20	6.8	21

FIGURE 3. Immunoprecipitation of Poly(A) Polymerase Activity Using Immune Serum. Poly(A) polymerase purified fractions from a phosphocellulose column (1.7 μg) and a heparin agarose column (0.3 μg) were mixed with immune and preimmune serum. Following a 3 h incubation at 4°C, 30 μl of a 1:1 suspension of Protein A-agarose were added and the incubation was continued for 2 h at 4°C. After centrifugation at 13,000 x g, the supernatants (20 μl) and pellets (10 μl) were assayed for poly(A) polymerase activity (see Materials and Methods and Appendix for further details of assays).

serum was used. A small amount of this activity was recovered in the immunoprecipitates, suggesting some reactivity of the antibody with non-essential epitopes.

Screening λgt11 Library with Polyclonal Antibody and Analysis of Positive Clones

A yeast genomic DNA library constructed in λgt11 was screened with the polyclonal antibody described above and nine positive clones were selected. Affinity-purification of the polyclonal antibody using the nine clones was carried out as described under Materials and Methods. The poly(A) polymerase peak fractions of a heparin agarose column (0.8 μg of fractions 20 and 21 combined) and a later fraction of the same column (20 μg of fraction 30 — this fraction was used in high concentration to test whether the contaminating protein band described in Fig. 1 reacted with a possible affinity-purified antibody) were spotted onto a strip of nitrocellulose and denatured with 25% isopropanol, 10% acetic acid. The strips were then washed in water, blocked with 5% Blotto-TBS, incubated with the possible affinity-purified antibody fractions and the reactivity of the protein spots was analyzed as described under Immunoscreening λgt11 Library (p.4). Fig. 4 shows that two of the λgt11 clones affinity-purified the polyclonal antibody.

DNA of the two positive λgt11 clones was digested with *Eco*R1, and a 2 kb insert fragment was detected by agarose gel electrophoresis for both, suggesting that the two were the same. By Northern blot analysis, the *Eco*R1 fragment reacted with both a 1.3 kb mRNA and a 1.8 kb mRNA (Fig. 5A). The 1.8 kb mRNA was of a size that could encode a 66 kDa protein, the size of the poly(A) polymerase band used for antibody production (Fig. 1).

Ligation of the λgt11 Positive Clone Insert into Low- and High-Copy Shuttle Plasmids

To test for possible overexpression of poly(A) polymerase activity, the 2 kb *Eco*RI endonuclease restriction fragment was ligated into the yeast centromere plasmid, pRS316, which was then transformed by electroporation into *E. coli* XL1-Blue and yeast strain YNN27. Crude extracts were

FIGURE 4. Analysis of Affinity-Purification of the Polyclonal Antibody by λgt11 Recombinants. Reactivity of (A) 0.8 μg of combined heparin agarose fractions 20 and 21 (see Fig. 1) and (B) 20 μg of heparin agarose fraction 30 (containing a contaminating protein band) with polyclonal antibody subjected to affinity-purification with the λgt11 positive clones. Heparin agarose fractions were applied to nitrocellulose, denatured, blocked to eliminate nonspecific reactivity, treated with antibody, and reactivity detected by AP secondary antibody — NBT/BCIP conjugate.

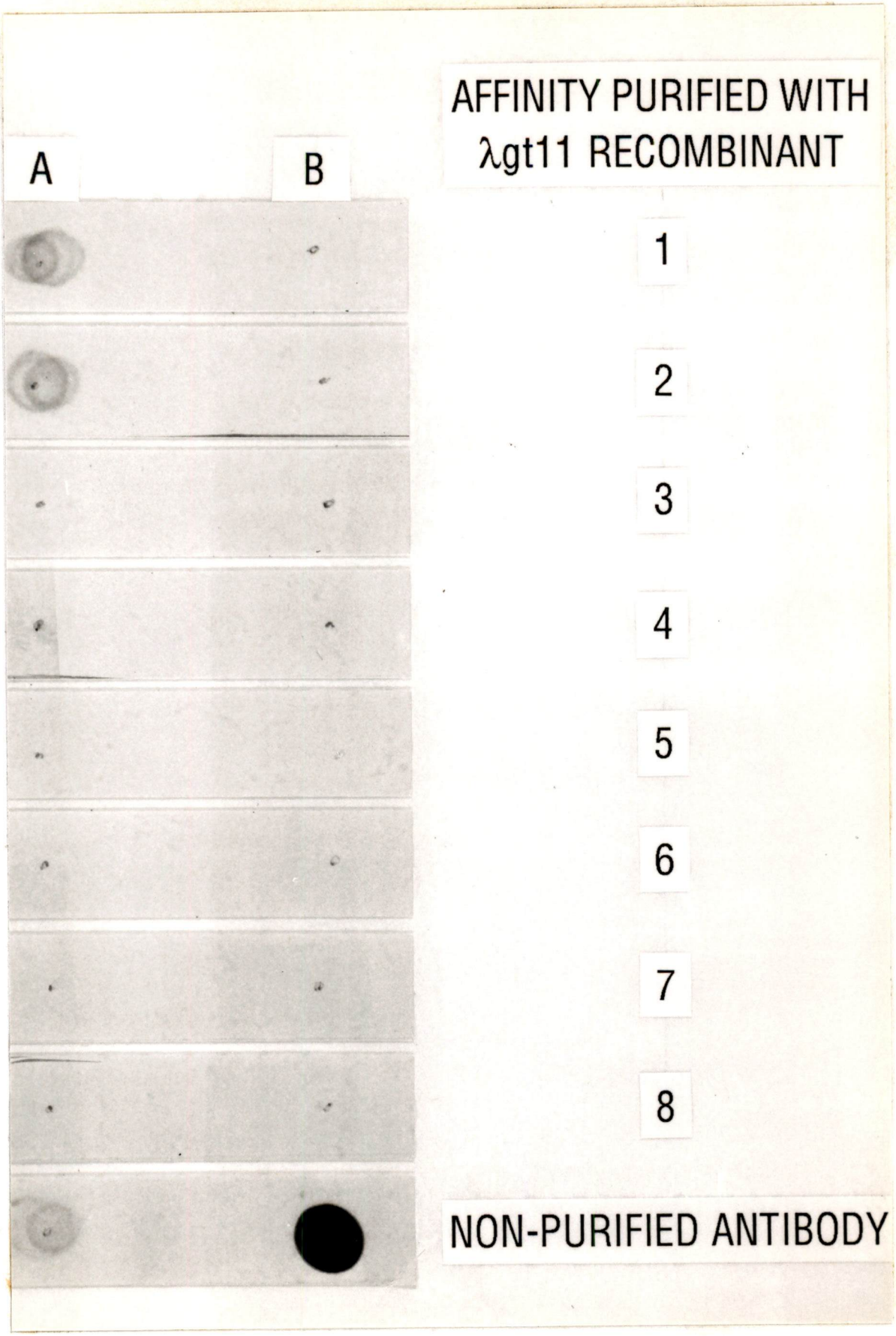
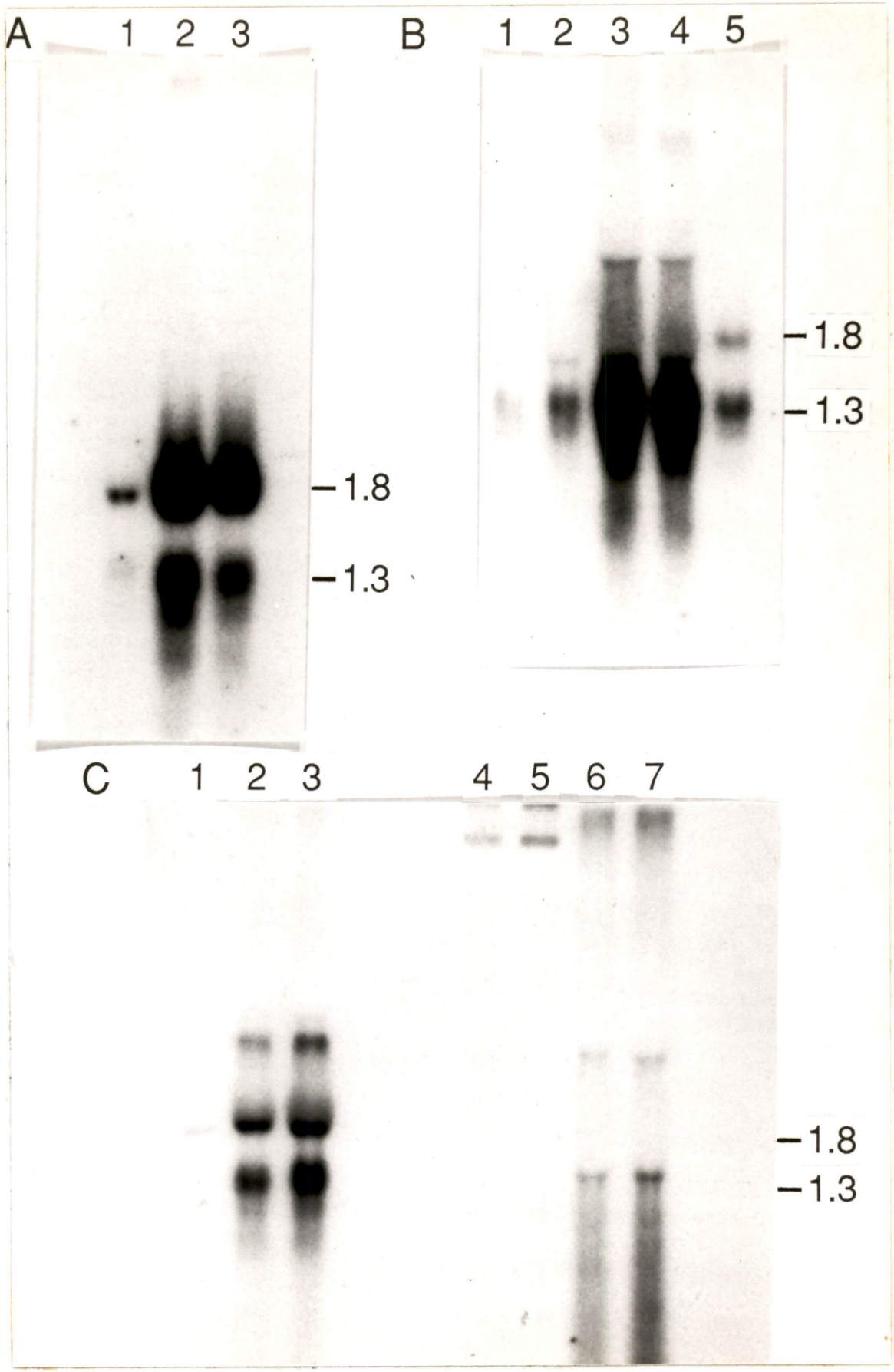

AFFINITY PURIFIED WITH
λgt11 RECOMBINANT
A
B
1
2
3
4
5
6
7
8
NON-PURIFIED ANTIBODY

FIGURE 5. Northern Analysis of RNA Fractions of Normal and Transformed Yeast and *E. coli.*

(A) The 2 kb DNA fragment of the λgt11 positive clone was used as a probe in the analysis. The RNA fractions were: Lane 1, 25 μg of YNN27 total RNA; lane 2, 4 μg of YNN27 mRNA; lane 3, 8 μg of YNN27 mRNA. RNA markers from BRL were used to size the RNA bands and sizes are shown at the right.

(B) A 1 kb *Hin*dIII fragment of the 2 kb insert in pRS316 was used as a probe for the following RNA fractions. Lane 1, 50 μg total RNA from wild-type yeast strain YNN27; lane 2, 30 μg of RNA from the strain transformed with pRS316 + clone insert; lane 3, 50 μg of RNA from the strain transformed with YEP24 + clone insert; lane 4, repeat of lane 3; and lane 5, 8 μg of YNN27 mRNA. The size of reactive bands are given on the right.

(C) The 2 kb *Sma*I/*Sal*I fragment of the pRS316 clone was used as a probe for the following RNAs: Lane 1, 30 μg of total RNA from yeast strain YNN27; lane 2, 15 μg of total RNA from the strain transformed with YEP24 clone; lane 3, 30 μg of the same RNA; lane 4, 30 μg of RNA from *E. coli* containing YEP24 plasmid; lane 5, 60 μg of the same RNA; lane 6, 30 μg of RNA from *E. coli* containing YEP24 clone; lane 7, 60 μg of the same RNA.


A
1 2 3
1.8
1.3
B
1 2 3 4 5
1.8
1.3
C
1 2 3
4 5 6 7
1.8
1.3

made of the *E. coli* and YNN27 cells, but no overexpression of poly(A) polymerase activity was detected (data not shown). By Northern blot analysis it could be shown that the 1.3 kb mRNA was overproduced (X2-3) but the 1.8 kb mRNA was not overproduced in the transformed YNN27 cells (Fig. 5B).

Because pRS316 is a single-copy number vector, it is unlikely to show a significant overexpression of the poly(A) polymerase gene. To further test overexpression by the clone, a 2 kb *SalI/SmaI* endonuclease restriction fragment was ligated into a high-copy number plasmid, YEP24. *E. coli* XL1-Blue and yeast strain YNN27 were transformed with the plasmid. No significant overexpression of poly(A) polymerase activity was measurable in extracts of the cells (data not shown). Northern blot analysis showed no overproduction of the 1.8 kb mRNA but a 20-fold overproduction of the 1.3 kb mRNA (Fig. 5B) in the transformed cells.

Selection of YEP24 Clones and Analysis of Poly(A) Polymerase Overexpression

In an attempt to obtain a yeast genomic DNA clone that caused overexpression of the 1.8 kb mRNA, presumably due to the presence of an intact poly(A) polymerase gene, a yeast genomic library containing larger DNA fragments in the high-copy number plasmid, YEP24, was screened by colony hybridization using the 2 kb *Eco*RI fragment as a probe. Two positive clones with inserts of 10-13 kb were obtained (called YEP24 clone #1 and clone #2), and yeast strain YNN27 was transformed with each of the plasmids. A Northern blot of RNA fractions of control yeast YNN27 and YEP24 clone #2 - transformed yeast is shown in Fig. 5C. Both the 1.3 kb mRNA and the 1.8 kb mRNA were overexpressed in the transformed cells. RNA of transformed *E. coli* was also examined, but no overexpression of the 1.8 kb mRNA was found. No significant overexpression of poly(A) polymerase activity was detected as shown in Fig. 6A. Both of the YEP24 clones showed the same results. Clone #2 was used in the further experiments (called YEP24 clone).

FIGURE 6. Analyses of Poly(A) Polymerase Overexpression by Activity Assays and by Western Blotting.

(A) Poly(A) polymerase activity assays with crude extracts of yeast strain YNN27 transformed with the control plasmid YEP24 and with the YEP24 clone. The poly(A) polymerase assay is described in Materials and Methods.

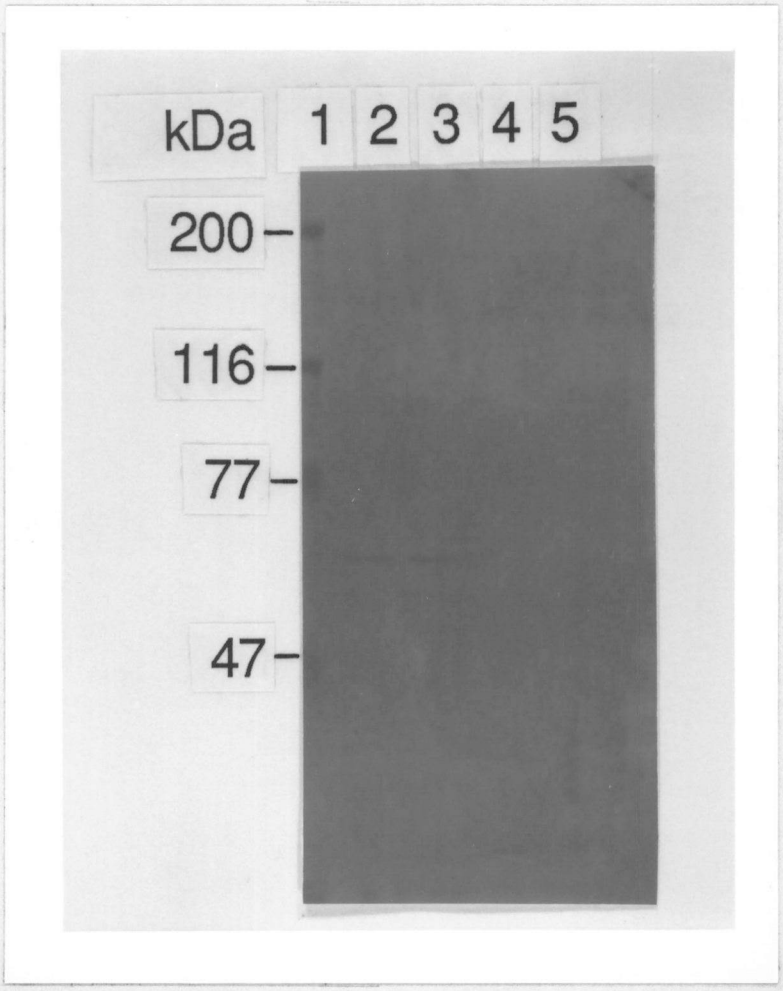
(B) Western blot analysis of crude poly(A) polymerase extracts using the affinity-purified antibody as a probe. From left to right, the lanes are: molecular weight standards with sizes in kDa given to the left of the blot, 400 μg of an extract of cells of YNN27 containing control plasmid YEP24, 400 μg of the same YNN27 containing the YEP24 clone, 700 μg of an extract of cells of *E. coli* containing control plasmid YEP24, 700 μg of the same of *E. coli* containing the YEP24 clone.

(A)

Assay of Yeast Strain YNN27 Spinco Supernatants

Supernatant	Volume	Poly(A) Polymerase cpm	Relative Activity Overexpression
YNN27 + YEP24	4 μl	1635	—
	8 μl	4392	—
YNN27 + YEP24 clone	4 μl	2033	x 1.24
	8 μl	5289	x 1.2

(B)



Western blot analysis of crude extracts of YNN27 and *E. coli* transformed with control plasmid YEP24 and with the positive YEP24 clone was carried out as described by Burnette (39). Affinity-purified antibody was used as a probe and antigen-antibody complexes were detected using alkaline phosphatase conjugated secondary antibody as described in Materials and Methods. Figure 6B shows that a 66 kDa protein band that reacts with the affinity-purified antibody is found in the crude extracts of yeast, but no overexpression of the protein occurred in the cells containing YEP24 clone. The extracts of *E. coli* showed no reactive protein.

Restriction Enzyme Mapping of the Clones

During the course of the studies just described, physical mapping of the 2 kb inserts and the YEP24 genomic DNA clones was carried out so an overlapping map of the genomic fragment could be constructed (49-51). Fig. 7 shows restriction maps of the clones. During the course of this investigation, it was learned that another laboratory had cloned a yeast poly(A) polymerase gene (57). Comparison of our restriction map with theirs showed the clones are not similar.

Use of the Affinity-Purified Antibody to Examine Its Reactivity with Specific Yeast Enzyme Fractions

Further analysis of yeast extracts was done to try to determine more about the reactivity of the affinity-purified antibody. It seemed possible that the antibody might also react with a second or third poly(A) polymerase such as species II and III described by Haff and Keller (21,22).

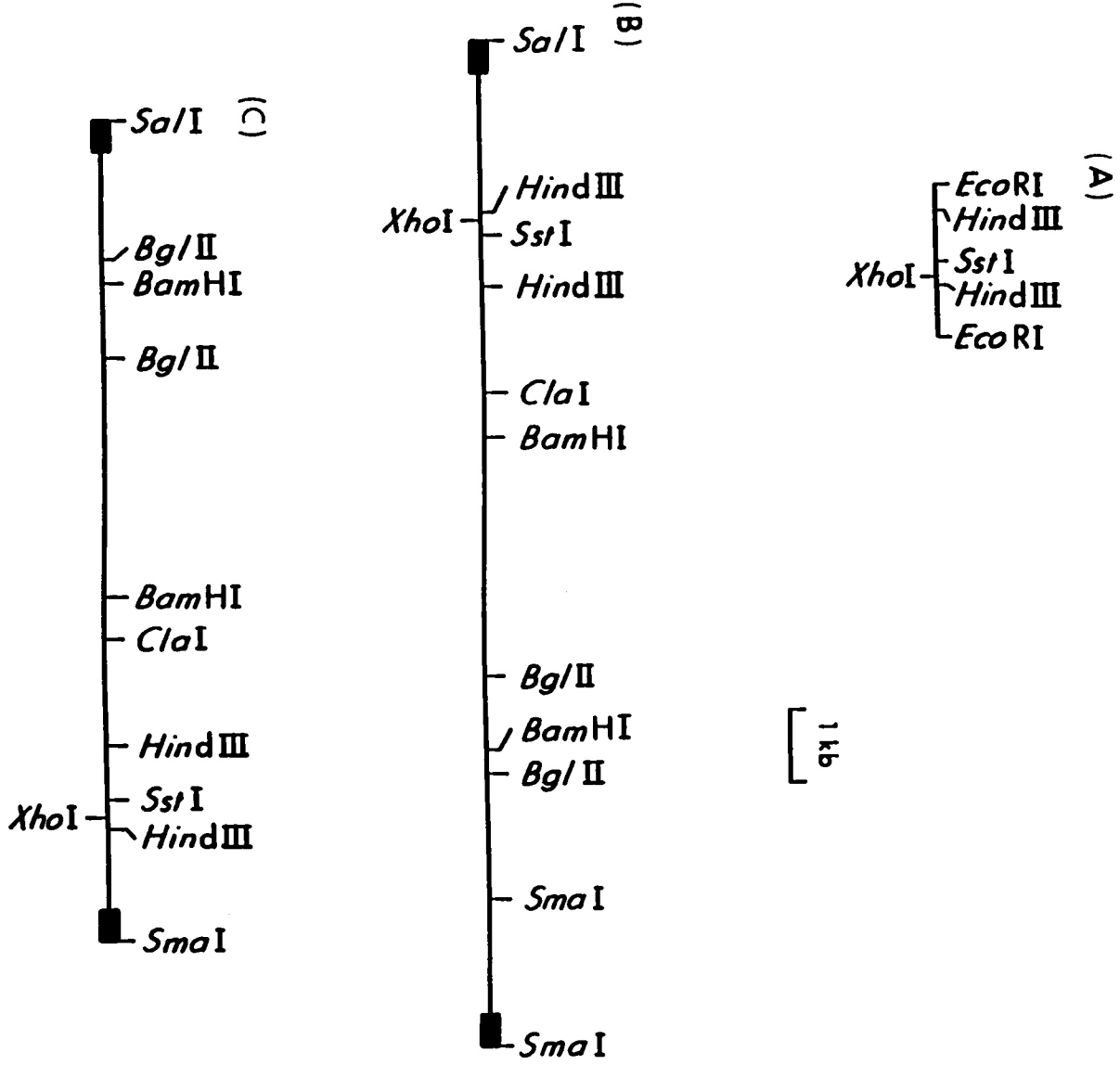
The affinity-purified antibody was used to analyze crude extracts of YNN27 and YNN27 containing a YEP24 clone. Fractions from DE-23 cellulose column chromatography of the extracts were also analyzed to try to detect an overexpressed reacting protein band. Crude extracts were prepared as described in Materials and Methods using PAP buffer and also with PAP buffer + .5 M KCl. A high-

FIGURE 7. Restriction Map of Positive Clones. Black bar indicates vector DNA; solid line indicates yeast DNA fragment inserted in the vector DNA.

(A) 2 kb *Eco*RI fragment of λgt11 clone.

(B) 10-12 kb *Sal*I/*Sma*I fragment of YEP24 clone #1.

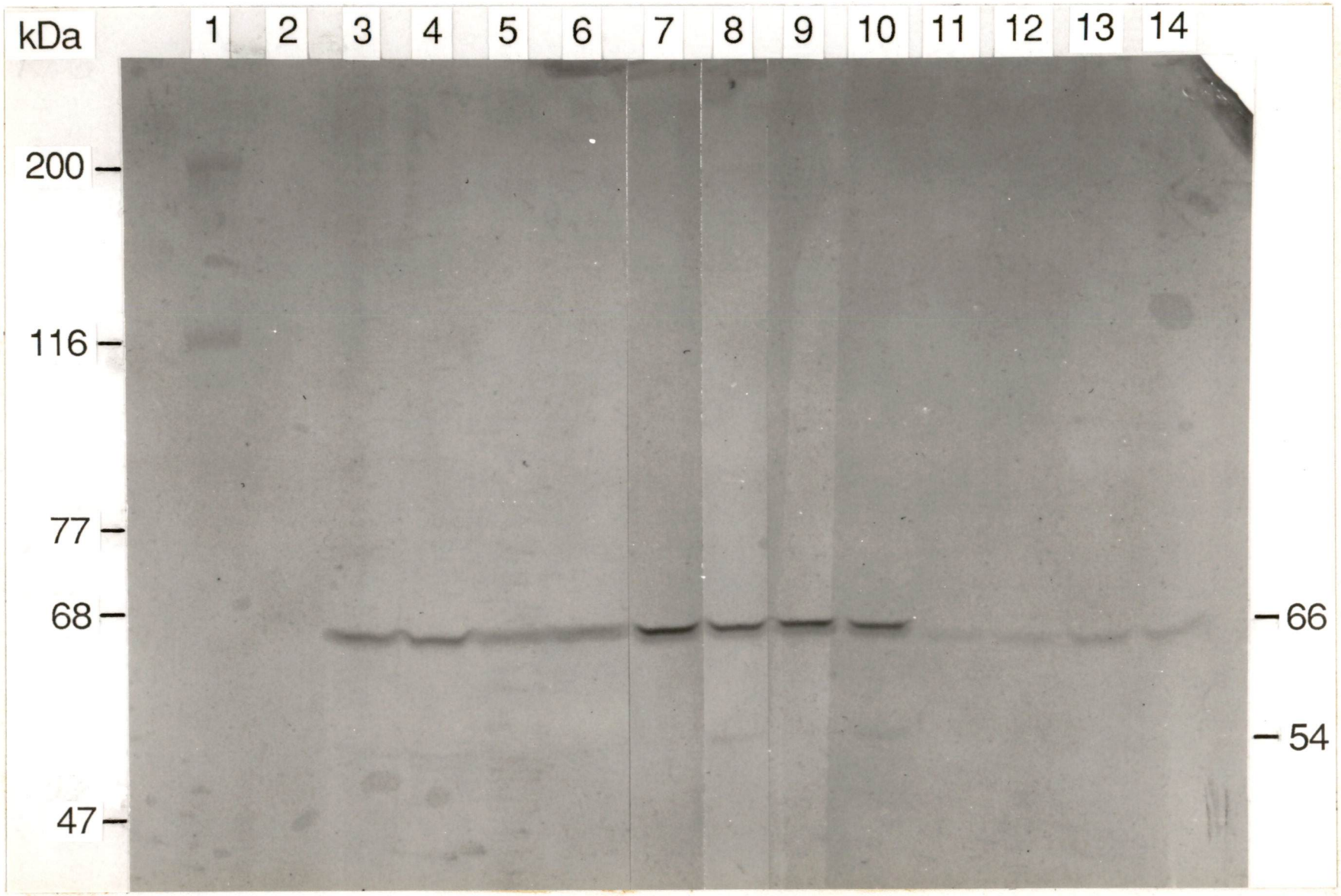
(C) 10-12 kb *Sal*I/*Sma*I fragment of YEP24 clone #2.


(C)
SalI
BglII
BamHI
BglII
BamHI
ClaI
HindIII
XhoI
SstI
HindIII
SmaI
(B)
SalI
XhoI
HindIII
SstI
HindIII
ClaI
BamHI
BglII
BamHI
BglII
SmaI
(A)
EcoRI
HindIII
XhoI
SstI
HindIII
EcoRI
1kb

salt wash was carried out on the ribosomes of the low-salt PAP extract using 20 mM Tris (pH 8.0), 0.5 mM EDTA, 0.5 M KCl, 4 mM $MgCl_2$, 0.2 mM DTT, 0.5 mM PMSF, 0.5 μg/ml leupeptin and antipain. The high-salt wash was dialyzed overnight against DE-23 buffer (p. 10) and then chromatographed on a DE-23 cellulose column. Fractions of 2 ml were collected, eluting first with DE-23 buffer + 0.1 M $(NH_4)_2SO_4$ and then with buffer + 0.3 M $(NH_4)_2SO_4$.

Western blot analyses of the enzyme fractions using the affinity-purified antibody is shown in Fig. 8. Two reacting bands of about 66 kDa are seen in the lanes containing the crude extracts (lanes 3-6), the high-salt ribosome wash fractions (lanes 7-8), and the DE-23 cellulose 0.1 M $(NH_4)_2SO_4$ fractions (lanes 9-10). The top band appears more in the ribosome high-salt wash fractions (lanes 7 and 8) and in the DE-23 cellulose –0.1 M $(NH_4)_2SO_4$ fractions (lanes 9-10), while the bottom band is seen more in the 0.35 M $(NH_4)_2SO_4$ elution of the DE-23 column (lanes 11-14). A third protein band at 54 kDa is found in the ribosome high-salt wash fractions and in the 0.1 M$(NH_4)_2SO_4$ elution of the DE-23 column (lanes 7-10). The 54 kDa protein band is present in higher amounts (X3-4) in the YEP24-transformed YNN27 fractions, and, thus, appears to be an overexpressed protein. The 54 kDa protein may possibly be a low level poly(A) polymerase encoded by the YEP24 clone, but it would be necessary to purify it more to test this possibility.

FIGURE 8. Western Blot Analysis of Protein Fractions of Control YNN27 and YEP24-Transformed YNN27. Crude extracts and partially purified poly(A) polymerase fractions of control YNN27 and YEP24-transformed YNN27 were electrophoresed on an 8% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with affinity-purified antibody. Lane 1, prestained molecular weight markers with the molecular weights indicated at the left; lane 2, albumin marker; lanes 3 and 4, 600 μg high salt extract (odd lanes are fractions from control YNN27, even lanes are fractions from YEP24-transformed YNN27); lanes 5 and 6, 600 μg low salt crude extract; lanes 7 and 8, 140 μg high salt wash fractions of ribosomes; lanes 9 and 10, 60 μg DE-23-0.1 M $(NH_4)_2SO_4$ peak fractions; lanes 11 and 12, 50 μg of DE-23-0.35 M $(NH_4)_2SO_4$ peak fractions; lanes 13 and 14, 100 μg of the same.


kDa
1
2
3
4
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6
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9
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11
12
13
14
200
116
77
68
47
66
54

CHAPTER IV

DISCUSSION

Rabbit polyclonal antibody was obtained by immunization with a gel protein band of approximately 66 kDa that paralleled poly(A) polymerase activity on purification. The immune serum that was obtained reacted with the 66 kDa protein band on Western blotting and immunoprecipitated poly(A) polymerase activity. On the basis of these properties, the antibody was used to probe a λgt11 yeast genomic library as the initial step in the cloning of a poly(A) polymerase gene. Nine positive λgt11 clones were obtained, and two were found to affinity-purify the antibody. The two clones were identical since *Eco*R1 digests showed both contained a 2 kb insert. The 2 kb fragment hybridized with a 1.3 kb and a 1.8 kb yeast mRNA when examined by Northern blotting. The 1.8 kb mRNA is of a size that could encode a 66 kDa protein. Yeast vectors were transformed with the 2 kb fragment of the λgt11 recombinant. Because overexpression of poly(A) polymerase activity was not seen, it seemed probable that only a portion of the poly(A) polymerase gene was present. The 2 kb fragment was used as a hybridization probe to probe a second yeast genomic library consisting of larger DNA fragments in the high expression vector, YEP24, in order to obtain an intact poly(A) polymerase gene. Two positive clones showed overexpression of both the 1.3 kb mRNA and the 1.8 kb mRNA, but they did not give any significant overexpression when assayed for poly(A) polymerase activity. Restriction maps of the two YEP24 clones were compared to each other and to a clone for a 63 kDa poly(A) polymerase cloned by Lingner, et. al. (57). Our restriction map showed no similarity to the map of that gene.

The results obtained suggested that our YEP24 clone might encode another poly(A) polymerase since Haff and Keller (21,22) had originally described three possible activities in yeast extracts.

Western blot analysis of yeast enzyme fractions obtained from cells containing the control plasmid YEP24 and the YEP24 clone was carried out to determine if the affinity-purified antibody could react with other protein bands in crude extracts and fractions obtained by DE-23 cellulose chromatography. The results of the analyses showed that the affinity-purified antibody reacted with two protein bands at about 66 kDa, one slightly smaller, but neither band was overexpressed by the YEP24 clone. The results also showed that there was overexpression of a protein band of 54 kDa that reacted with the affinity-purified antibody. Studies in other laboratories show that poly(A) polymerases with molecular weights ranging from 50,000 - 84,000 have been found (20,53,54).

Overall, the results suggest that the 54 kDa protein is encoded by our clone, and it may be a low level poly(A) polymerase or a protein that contains an epitope recognized by the antibody. The affinity-purified antibody may be useful in further analyses of whether yeast contains several poly(A) polymerases. Three proteins react in Western blotting, but it will require considerably more purification of these proteins to determine their relatedness. Sequencing of the YEP24 clone also may be carried out in the future to determine if it is homologous to clones of poly(A) polymerases of higher eukaryotes, one of which, namely a bovine poly(A) polymerase, has very recently been described (55).

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APPENDIX

SM

10 mM Tris-HCl, pH 7.5

10 mM $MgCl_2$

LB Media

10 g/l Bacto-tryptone

5 g/l Bacto-yeast extract

10 g/l NaCl

LB plates include 15 g/l Bacto-agar

Top agar includes 7.5 g/l Bacto-agar

STE

10 mM Tris-HCl, pH 8.0

100 mM NaCl

1 mM EDTA, pH 8.0

TBE (5×)

54 g/l Tris base

27.5 g/l boric acid

20 ml/l 0.5 M EDTA, pH 8.0

YPD Media

10 g/l Bacto-yeast extract

20 g/l Bacto-peptone

20 g/l Dextrose

SC (25×)

Adenine	240 mg
Arginine	"
Histidine	"
Leucine	360 mg
Lycine	"
Methionine	"
H_2O	360 ml

SC Plates (1 liter)

40 ml	SC stock (25×)
20 g	Dextrose
6.7 g	Yeast Nitrogen Base
20 g	Bacto Agar
960 ml	H_2O

After sterilization, add filter-sterilized threonine (7.5 ml of 20 mg/ml) and tryptophan (10 ml of 2 mg/ml).

PAP Buffer

0.02 M Tris, pH 8.0

0.5 mM EDTA

0.005 M $MgCl_2$

1 mM DTT

1 mM PMSF

1 μg/ml leupeptin

0.5 μg/ml antipain

PAP Reaction Mix (20×)

0.04 ml 0.01 M ATP

0.02 ml ICN [^{3}H]-ATP

0.10 ml 1 M Tris, pH 8.5

0.02 ml 0.1 M $MnCl_2$

0.3 ml rRNA

0.1 ml 0.1 M mercaptoethanol

Poly(A) Polymerase Assay

Parameter	Value
Reaction Volume	0.1 ml
Temp.	30°C
Time	30 min
PAP Reaction Mix (20×)	0.03 ml
Enzyme	0.1-1 mg/ml

Reactions are terminated by the addition of 1 ml ice cold 5% TCA, 0.1 M sodium pyrophosphate (NaPPI). After 5 min on ice, the reaction is poured onto Whatman GF/A glass fiber filters, and vacuum is applied as the filter is then washed 5 times with 4 ml portions of the cold 5% TCA, 0.01 M NaPPI. The filters are dried, transferred to a scintillation vial, and after addition of 10 ml Beta Max (ICN) radioactivity is measured.

BPB

50%	Glycerol
100 mM	EDTA, pH 8.0
0.25%	Bromophenol blue

Prehybridization Solution

50%	Deionized formamide
6×	SSPE
0.05×	Blotto

1× Blotto

5% nonfat dried milk dissolved in water containing 0.02% sodium azide

High-Salt Wash Buffer

20 mM Tris, pH 8.0

0.5 mM EDTA

0.5 M KC1

4 mM $MgC1_2$

0.2 mM DDT

0.5 mM PMSF

0.5 μg/ml antipain

0.5 μg.ml leupeptin

Immunoprecipitation of Poly(A) polymerase

5 μl	Peak fraction
50 ml	Buffer A
5-10 μl	Immune sera or preimmune sera

Incubate 3h in cold room – shaking occasionally.

Add 30 μl protein A agarose (50% in Buffer A).

Incubate 2 h in cold room, rocking

Spin 5 min – Remove supernatant and save for assay.

Wash beads by adding 0.5 ml Buffer A and vortex to mix well.

Spin – Discard supernatant and wash again.

Suspend pellet in 15 μl Buffer A.

Assay supernatant and pellet for PAP activity.

Buffer A

10%	Glycerol
0.05 M	Tris, pH 7.9
0.1 M	KCl
1 mM	EDTA
0.05%	NP40

Immediately before using add:

1 mM	DTT
0.5 mM	PMSF
0.5 μg/ml	leupeptin
0.5 μg/ml	antipain

VITA

Marilyn Maupin was born January 11, 1951 in Rockwood, Tennessee. She attended Pine Orchard Elementary School and Oakdale High School. She received her Bachelor of Science degree in Biology in 1971 and her Master of Arts degree in Education in 1981 from Tennessee Technological University. Ms. Maupin taught in the Morgan County School System from 1971 to 1980. She began her present employment with Oak Ridge National Laboratory in 1980.