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Structural Modifications of RNA Influence the 5′ Exoribonucleolytic Hydrolysis by XRN1 and HKE1 of *Saccharomyces cerevisiae*

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Two 5′ exoribonucleases, XRN1 and HKE1, of *Saccharomyces cerevisiae* have been found to have very important cellular roles, XRN1 playing a key role in mRNA turnover and HKE1 in pre-rRNA processing. Here, an analysis of strong secondary structures in RNA that cause blocks or stalls (accumulation of RNA fragments that are shortened from the 5′ end to the site of the secondary structure insertion) in the processive exoribonucleolytic hydrolysis reactions is reported. With both enzymes, oligo(G) tracts of lengths 18, 16, and 9 stall quite effectively, and the stalls are close to the start of the oligo(G) stretch. Two strong stem-loop structures cause measurable but low-level stalls with both enzymes. If the stem-loop structure is placed close to the 5′ end of the RNA, substantial inhibition of overall RNA hydrolysis occurs with HKE1 and less, but measurable, inhibition with XRN1. RNA structural modification caused by protein complexing has been investigated by using poly(A) binding protein. The hydrolysis of poly(A) by XRN1 is inhibited by poly(A) binding protein, while HKE1 activity is not affected.

Two 5′ exoribonucleases, termed XRN1 and HKE1 according to their gene designations, were both highly purified and characterized in this laboratory [1-3]. They catalyze a processive hydrolysis of RNA from the 5′ end with the production of 5′-mononucleotides. The properties of the two enzymes are very similar, both being most active on RNAs that contain a 5′ phosphomonoester end group and inhibited by 5′-end modifications such as a cap structure or a 5′-triphosphate end group. Their relative activities with RNA and ssDNA are also similar with both hydrolyzing DNA at about 5-8% of the rate of RNA. The amino acid sequences of the two proteins show significant homology, as reported by Amberg et al. [4] and Aldrich et al. [5].

The biological functions of the two proteins have been analyzed in some detail following the cloning of the genes encoding them. The cloning of the *XRN1* gene in this laboratory showed the gene, encoding a 175 kDa protein, was not essential; however, its disruption led to disparate phenotypes and slow cell growth [6, 7]. The gene has also been isolated in five other laboratories by unrelated approaches (see review by Kearsey and Kipling [8] and Liu and Gilbert [9]). Other gene designations include *SEP1* [10], *DST2* [11], *KEM1* [12], and *RAR5* [13]. The activity of the enzyme as both a 5′ exonuclease and a DNA strand exchange protein has been studied in some detail [14,15]. Heyer et al. [16] have reported that the protein is a cytoplasmic protein. Analysis of its effect on RNA metabolism in this laboratory showed that cells in which the gene was disrupted accumulated an internal transcribed spacer fragment of pre-rRNA as well as short-lived mRNAs that lacked both the poly(A) tail and the cap structure [17,18]. The latter finding strongly suggested that XRN1 was involved in mRNA turnover and that it hydrolyzed the mRNA following its deadenylation and decapping. Decker and Parker [19] showed at this time that if a poly(G) sequence was inserted into *MFA2* mRNA, RNA fragments consisting of the G tract and downstream sequences accumulated during the mRNA turnover process due to an apparent block of 5′-exoribonuclease action. In cells depleted of the *XRN1* gene, the fragments were not found [20], suggesting the role of the encoded protein in mRNA turnover. The overall role of XRN1 in the mRNA turnover pathway has been reviewed recently by Caponigro and Parker [21].

The HKE1 gene was cloned in three laboratories [4, 5, 22] and found to encode a 116 kDa protein. The gene

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Abbreviations: XRN1, protein encoded by the *XRN1* gene of *S. cerevisiae;* HKE1, protein encoded by the *HKE1* gene of *S. cerevisiae;* PABP, poly(A) binding protein.

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is essential and has been implicated in several nuclear functions including nuclear export of mRNA [4] and generation of 5.8S rRNA during pre-rRNA processing [4, 23].

Since the two 5′ exoribonucleases play very important functional roles, it was of interest to determine the extent to which structural modifications of an RNA molecule affect hydrolysis with the highly-purified enzymes. Such modification may be of significant use in the future for studies involving the mechanisms and control of RNA turnover and processing. Results with both XRN1 and HKE1 are reported here.

MATERIALS AND METHODS

Plasmids. pRP490 and pRP513 were the kind gifts of Denise Muhlrad and Roy Parker (Univ. of AZ, Tucson). pRP490 contains two oligo(G)18 tracts at nucleotides 28 and 178 of the *MFA2* RNA sequence, while pRP513 contains none [20]. In order to put oligo(G)16 and oligo(G)9 tracts into the *MFA2* RNA, a *Bgl*II site was put into pRP513 by oligonucleotide-directed mutagenesis [24]. The *Bgl*II site was placed at nucleotide 27 of the *MFA2* RNA sequence using the following oligonucleotides: TP005 5′-AACAAGATCTACCAACCT-TAATG-3′, and TP006 5′-GTATGAAGATGATAGCTCGCT-3′. This placed the *Bgl*II site about 300 nucleotides downstream from the SP6 promoter. The resulting plasmid was referred to as p513Bg1. Plasmid pG16 was created by digesting p513Bg1 with *Bgl*II and inserting the following annealed oligonucleotides: TP001 5′-GAT-CTAGGAATTTGGGGGGGGGGGGGGGAATTCCT-3′, and TP002 5′-GATCAGGAATTCCCCCCCCCCCCCCCAAATTCCTA-3′. Plasmid pG9 was created in the same manner using the annealed oligonucleotides: TP003 5′-GATCTAGGAATTTGGGGGGGGGAATT-CCT-3′, and TP004 5′-GATCAGGAATTCCCCCCCCCAAATTCCTA-3′. Restriction sites in these plasmids downstream of the oligo(G) tracts included *Sma*I, *Bam*HI and *Eco*RI [20].

A series of plasmids containing a sequence capable of forming a stem-loop structure previously described by Vega Laso et al. [25] were made using the following annealed oligonucleotides: TP010 5′-GATCTACCAGCTTACGCCCGCCAAACAGGCGGGCGTAAGCTG-TGAATTCAT-3′ and TP011 5′-GATCATGAATTCACAGCTTAC-GCCCGCCTGTTTGGCGGGCGTAAGCTGGTA-3′. Plasmid pST300 was constructed by cutting p513Bg1 with *Bgl*II and ligating the stem-loop oligonucleotides into this site. Two additional plasmid constructs containing the stem-loop sequence were made by deleting the 300 base region upstream of the *MFA2* RNA coding region. This was done by digesting pST300 with *Hind*III and *Bgl*II, then gel purifying the plasmid to remove the 300 base fragment. The plasmid was then digested with mung bean nuclease (Gibco BRL) and ligated to create pST9 in which the stem-loop structure was nine nucleotides from the transcription start site of the SP6 promoter. Alternatively, the digested and gel-purified pST300 was incubated in a Klenow reaction to fill in the single-stranded ends, then ligated to create pST20. In this construct, the stem-loop structure was 20 nucleotides from the transcription start site. A plasmid referred to as p513d was also made by ligating *Hind*III, *Bgl*II-cut p513Bg1 following mung bean nuclease trimming. This plasmid, lacking a stem-loop structure, was used as a control. The plasmid pBS5Bam was obtained from Dr. Robert Schneider (New York Univ. Med. Center). This plasmid contains a 70 nucleotide sequence containing 5 ligated *Bam*HI linkers in the *Eco*RI and *Nhe*I site of Bluescript II SK (Stratagene). An *Eco*RI, *Sac*I fragment of this plasmid was inserted into the same sites of pSP65 (Promega) to construct pST#2, a DNA used to make RNA with a second stem-loop structure. All procedures used in the constructions just described were as described in Sambrook et al. [26] and all the DNAs were sequenced to verify that the constructions were correct. All oligonucleotides were obtained from Integrated DNA Technologies.

Purification of XRN1 and HKE1. XRN1 was purified according to the procedure of Johnson and Kolodner [14] using the high expression vector described by them [kindly provided by Arlen Johnson (Univ. of TX, Austin)]. HKE1 was purified as recently described [3].

Preparation of RNA substrates. DNAs were prepared using the Qiagen Maxi system. Usually, 200-500 μg of DNA were then purified further using FPLC chromatography on MonoQ columns. After suitable restriction cleavage of the plasmids as described in each Figure legend, the RNAs were transcribed using the Riboprobe Gemini II Transcription System (Promega) with SP6 RNA polymerase and [α-^{32}P]GTP (DuPont). The 5′-triphosphate terminus of each transcript was hydrolyzed to a 5′-monophosphate terminus by incubation of approximately 2 nmole of RNA with 30 units of tobacco acid pyrophosphatase (Epicentre Technologies) in a 150 ul reaction mixture according to the manufacturer's directions. The RNAs were then purified by electrophoresis on a polyacrylamide-urea gel followed by excision and elution of the desired band from the gel with Probe Elution Buffer (Ambion). Following phenol-chloroform extraction, the RNAs were ethanol-precipitated, washed and dried. The oligo(G)-containing RNAs were dissolved in water [50 μl plus 80 units of RNasin (Promega)]. The RNAs containing a stem-loop structure were dissolved in 100 μl of 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 50 mM NaCl, and 0.1% SDS. The RNAs then were heated at 90°C for 4 min and placed at 65°C to slow cool to 40°C. The RNAs were recovered by ethanol precipitation followed by washing and drying and dissolved in water as described just above.

Exoribonuclease reactions and analysis. The exoribonuclease reaction mixtures (50 μl) contained: [^{32}P] RNA, 0.3 μg (1×10^5 cpm), 33 mM Tris-HCl buffer, pH 8.0, 2 mM $MgCl_2$, 50 mM NH_4Cl, 0.5 mM DTT, 30 μg of acetylated albumin (BRL), 80 units of RNasin, and the amount of enzyme described in the Figure legends. The mixtures were incubated for 10 min at 37°C, and the reactions were stopped by the addition of SDS to 0.1%. Aliquots of the mixtures were then analyzed by polyacrylamide gel electrophoresis. In the cases in which 5′-GMP formation was measured, 7 μl of the mixtures were applied to Whatman 3MM paper and high voltage electrophoresis was carried out at 2000V for 45 min using pyridine-acetate buffer (pH 3.5). The GMP bands were excised and the radioactivity measured. [3H]Poly(A) was prepared as previously described [1] and PABP was a kind gift of Allan Jacobson (University of Massachusetts Medical Center, Worchester). The reactions with poly(A) were carried out and analyzed as described recently [3].

RESULTS AND DISCUSSION

Modification of the structure of mRNA by insertion of oligo(G) tracts has been shown to influence mRNA turnover in S. *cerevisiae.* Vreken and Raué [27] showed that mRNA turnover in vivo could be blocked by an oligo(G)18 tract presumably due to blockage of a 5′ exoribonuclease, and Decker and Parker [19] and Muhlrad et al. [20] showed the same result and that the enzyme involved was XRN1. RNA fragments shortened from the 5′ end to the site of the secondary structure insertion accumulated in the yeast cells. Poly(G) and oligo(G)-rich polymers form unusual structures that may involve both intra- and intermolecular interactions and the formation of G-quartet structures [28,29]. Using the two oligo(G)18-containing *MFA2* mRNA con-

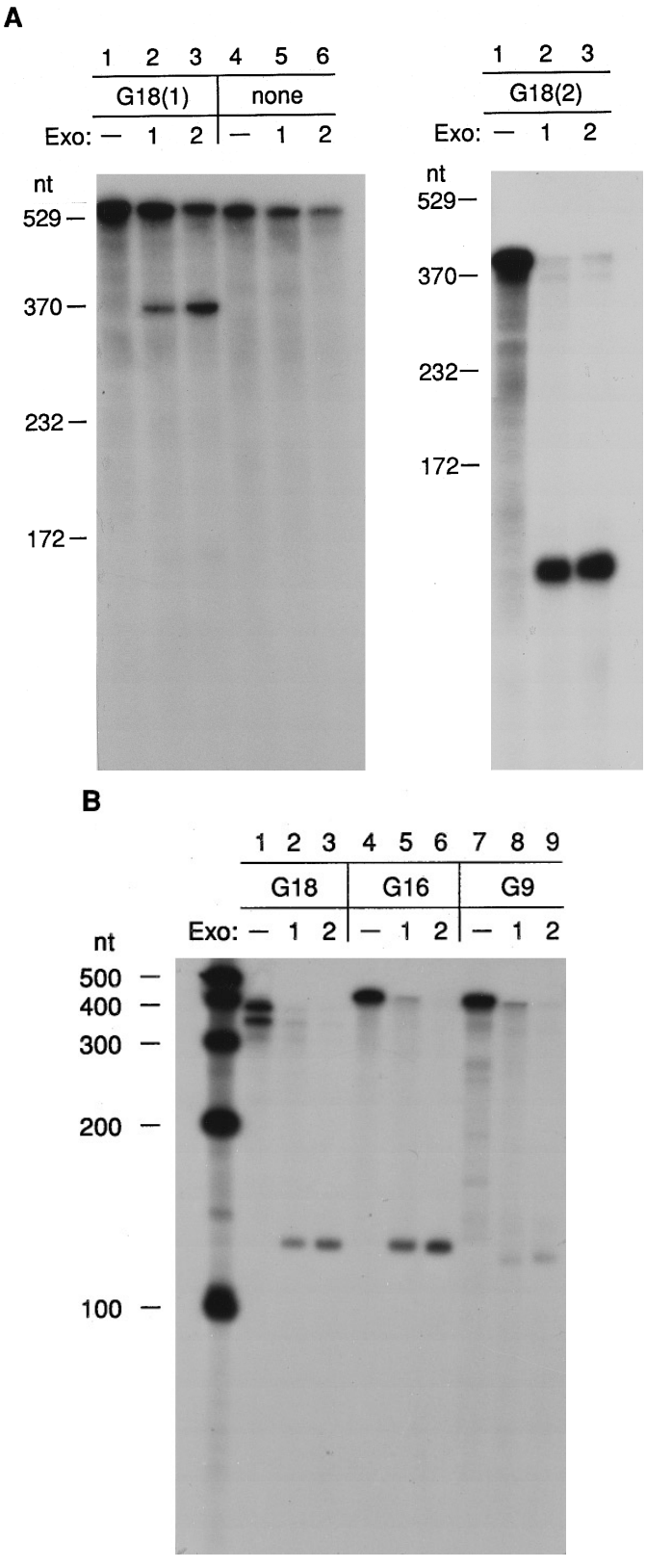

FIG. 1. Analysis of XRN1 and HKE1 hydrolysis products from oligo(G)-containing RNAs. (A) For the synthesis of oligo(G)18(1) RNA and the control RNA lacking an oligo(G) tract (none), pRP490 and pRP513, respectively, were cut with *Sma*I. For oligo(G)18(2) RNA, pRP490 was cut with *Bam*HI. XRN1 is Exo: 1 and HKE1 is Exo: 2. At the left, lanes 1, 2, and 3, oligo(G)18(1) RNA was incubated with no enzyme, XRN1 (4.4 units), and HKE1 (52 units). Lanes 4, 5, and 6 are the same with the control RNA. For oligo(G)18(2) RNA at the right, the enzyme amounts were the same. (B) pRP490, pG16, and pG9 DNAs were linearized with *Bam*HI for transcription. For each oligo(G) RNA, the amounts of the enzymes were the same as in (A). The digestion products were analyzed on 6% polyacrylamide gels (1.5mm) with 6M urea.

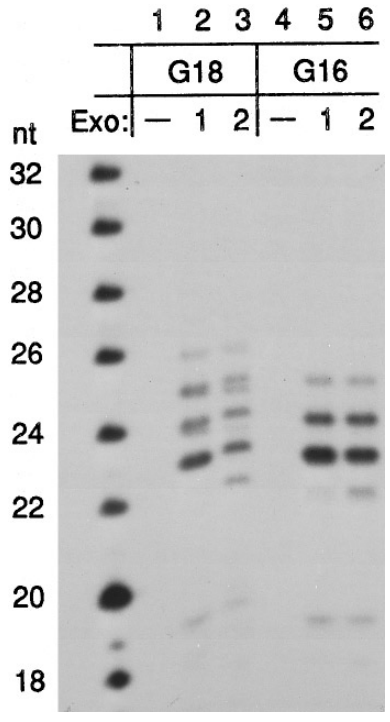

FIG. 2. Analysis of the site of the stall with XRN1 and HKE1 using oligo(G)18 and oligo(G)16 RNAs. The plasmids pRP490 and pG16 were linearized with *Eco*RI for transcription. Digestions with XRN1 and HKE1 were as described in the legend to Fig. 1, and the products were run on a 12% polyacrylamide gel (0.4mm) with 7M urea at 55°C.

structs of Mulhrad et al. [20] and a control construct lacking the (G)18 tract, Stevens and Poole [3] briefly described stalling in vitro with the two exoribonucleases. The in vitro results showed the accumulation of RNA fragments of the expected size. To establish the nature of the oligo(G) tract stalls and compare the two enzymes, further analyses were done, as shown in Fig. 1 and Fig. 2. First, one of the oligo(G)-containing DNAs was cut at two restriction sites for transcription, and oligo(G)-containing RNAs of different length were prepared and analyzed as shown in Fig. 1A. (G)18 RNA(1) contains the (G)18 tract about 370 nt from the 3′ end of the RNA and a fragment of such a length accumulates highly upon incubation with both en-

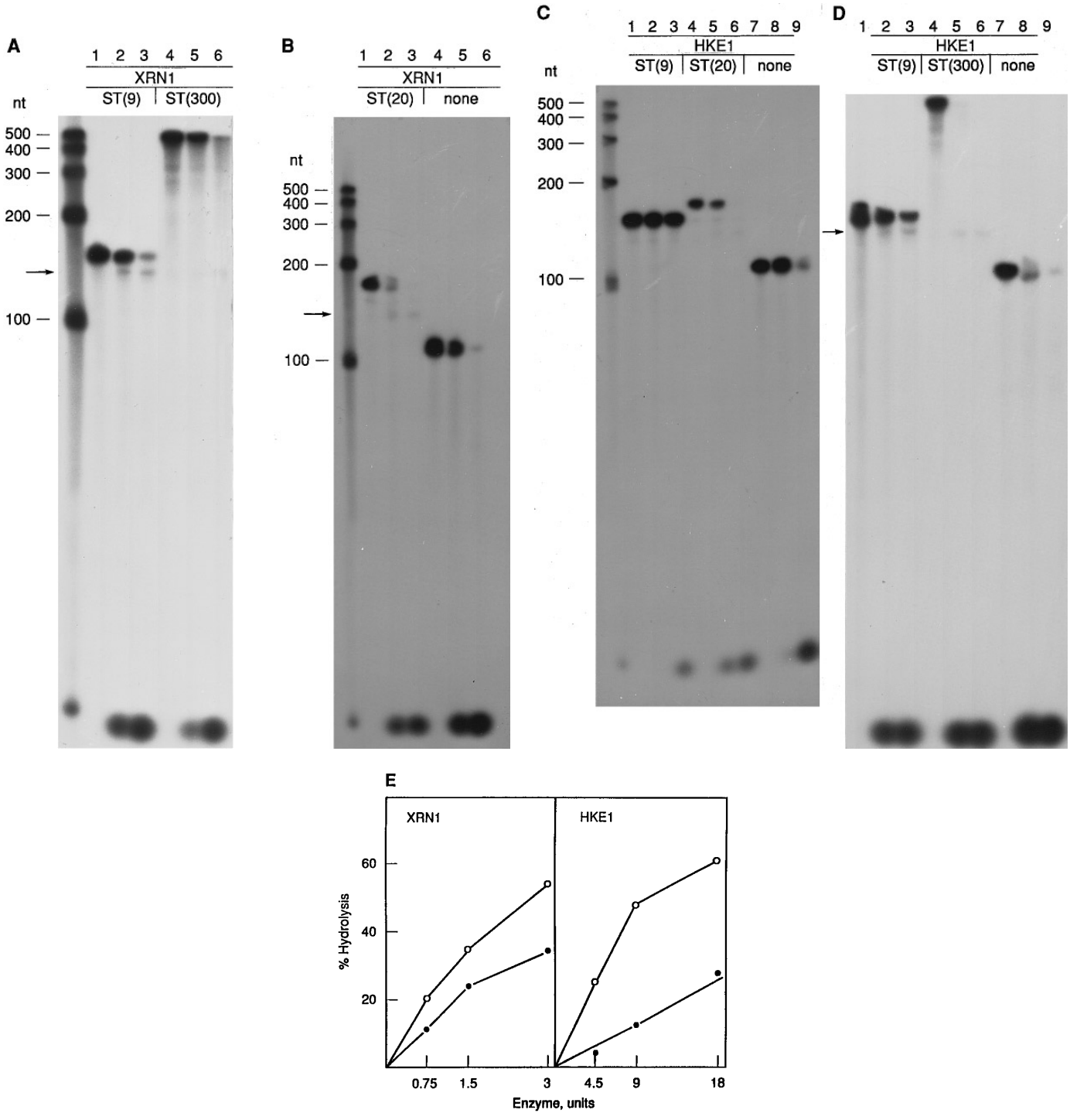

FIG. 3. Analysis of XRNI (A and B) and HKE1 (C and D) hydrolysis of RNAs containing a stem-loop structure at different distances from the 5′ end. The RNAs labeled ST9, ST20, ST300, and "none" are the products of transcription of pST9, pST20, pST300, and p513d linearized with *Bam*HI. The concentrations of XRN1 in (A) and (B) were lanes 1 and 4, no enzyme; lanes 2 and 5, 2.5 units; and lanes 3 and 6, 7.5 units. The concentrations of HKE1 in (C) were lanes 1, 4, and 7, no enzyme; lanes 2, 5, and 8, 6 units; and lanes 3, 6, and 9, 18 units. The concentrations in (D) were lanes 1, 4, and 7, no enzyme; lanes 2, 5, and 8, 8 units; and lanes 3, 6, and 9, 24 units. The reaction mixtures were analyzed on 6% polyacrylamide gels (1.5 mm) containing 6M urea. Arrows denote stall fragments. (E) shows the percentage hydrolysis of the ST9 (solid circles) and ST20 RNA (open circles) with different amounts of XRN1 and HKE1as measured by 5′-GMP formation.

zymes (see lanes 1-3). RNA containing the G18 tract about 130 nt from the 3′ end [(G)18 RNA(2)] yielded a shorter fragment of approximately 130 nt, again with both enzymes. The results show that RNA fragments accumulate that are shortened from the 5′ end to the site of the secondary structure insertion. The results of the experiments showed the fragments are formed at the same rate as GMP and that they are only poorly hydrolyzed further (data not shown).

The length of the oligo(G) tract needed for stalling of the 5′ exoribonucleases in vitro was analyzed by using RNAs with oligo(G)16 and oligo(G)9 tracts. The effects of the oligo(G)16 and oligo(G)9 tracts in comparison to the oligo(G)18 tract are shown in Fig. 1B. At complete hydrolysis of the substrate RNA, the amount of the stall fragment was determined by densitometer scanning of the films and calculations on the basis of the G content of both the RNA and the stall fragment. The oligo(G)18 and oligo(G)16 tracts both caused greater than 90% accumulation of an unhydrolyzed 3′ RNA fragment. The oligo(G)9 tract showed a more moderate effect, measured at 10-15% accumulation of a fragment of the expected size.

The results in Fig. 1 showed the size of the stall fragment was close to that predicted. The RNA containing an oligo(G)9 tract yielded a slightly shorter fragment than RNAs with an oligo(G)18 or oligo(G)16 tract. RNAs containing the oligo(G)18 and the oligo(G)16 tracts four nucleotides from the 3′ end of the RNA were hydrolyzed by both enzymes and the accumulated RNA fragments were sized. The results are shown in Fig. 2. The fragments that accumulate with both RNAs and both enzymes range in size from 22-26 nucleotides when compared to the oligo(dN) marker oligonucleotides. The different sizes of the fragments may be due to different stall sites or to transcription termination at different sites. The size of the stall fragments was in a range that showed that the exoribonucleases are blocked close to the start of the oligo(G) tract.

The influence of strong stem-loop structures in RNAs upon the hydrolysis by XRN1 and HKE1 was also measured. First, a stem-loop structure with a predicted ΔG of approximately -28 kcal/mol was inserted into the same *MFA2* RNA construct as was used with the oligo(G)-containing RNAs. In vivo, this stem-loop structure blocks translation initiation highly [25]. With the two enzymes a low-level accumulation of RNA stall fragments (approximately 5% of substrate RNA) could be detected when the RNA (ST 300) was highly degraded. RNAs were also synthesized so that the stem-loop structure was 9 and 20 nucleotides (ST9 and ST20 RNAs) from the 5′ end of the RNA rather than 300 nucleotides as in the *MFA2* RNA construct. This was done to compare blockage of overall RNA hydrolysis due to inhibition of enzyme binding or initiation of hydrolysis. The results on the overall rates of hydrolysis and the amounts of RNA 3′ fragments accumulated with ST9, ST20, and ST300 RNAs are shown with different concentrations of XRN1 in Fig. 3A and 3B and for HKE1 in Fig. 3C and 3D. In all cases, a small amount of accumulated fragments was detected. The ST(9) RNA results with both enzymes show the stall fragment best (see lanes 2 and 3 in Fig. 3A and lanes 2 and 3 in Fig. 3D). However, with HKE1, the stem-loop structure at 9 nucleotides from the 5′ end was quite inhibitory to the overall hydrolysis of the RNA. The rates of hydrolysis of RNAs with the structure at both 9 and 20 nucleotides from the 5′ end were also compared by measuring 5′ GMP formation at several enzyme concentrations. A comparison of these two RNAs was done since they differ in length and sequence by only 11 nucleotides. These results are plotted in Fig. 3E, and they show that with HKE1, the rate of hydrolysis of the RNA with the structure at 9 nucleotides is only about 20% of that of the RNA with the structure at 20 nucleotides. As shown, XRN1 is inhibited less by the stem-loop structure at 9 nucleotides, about 30-40%.

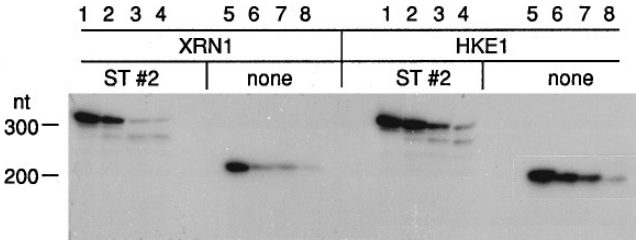

FIG. 4. Analysis of XRN1 and HKE1 hydrolysis of RNA containing a second strong stem-loop structure. pST#2 and pSP65 were linearized with *Pvu*II for transcription of the RNA called ST#2 (second stem-loop structure) and the control RNA ("none," no stem-loop structure). The amounts of enzymes used were XRN1: lanes 1 and 5, no enzyme; lanes 2 and 6, 2 units; lanes 3 and 7, 4 units; and lanes 4 and 8, 8 units. For HKE1, the amounts were lanes 1 and 5, no enzyme; lanes 2 and 6, 6 units; lanes 3 and 7, 12 units; and lanes 4 and 8, 24 units. The reaction mixtures were analyzed on 6% polyacrylamide gels (0.4mm) plus 6 M urea.

A stronger stem-loop structure ($\Delta G = -80$ kcal/mol) was described by Curatola et al. [30] as useful in blocking mRNA turnover when located in mRNAs anywhere 5′ of an AU-rich destabilizing element (the granulocyte-macrophage colony-stimulating factor ARE). An RNA construct containing this stem-loop structure about 20 nucleotides from the 5′ end of the RNA was also analyzed for stalling of the exoribonucleases. The results of its hydrolysis as compared to RNA lacking the structure are shown in Fig. 4 with both XRN1 and HKE1. Again, a low-level of stall fragment (measured at about 5-8% of the initial substrate RNA) was detected with both enzymes. The overall rate of hydrolysis of the RNA containing the structure, as measured

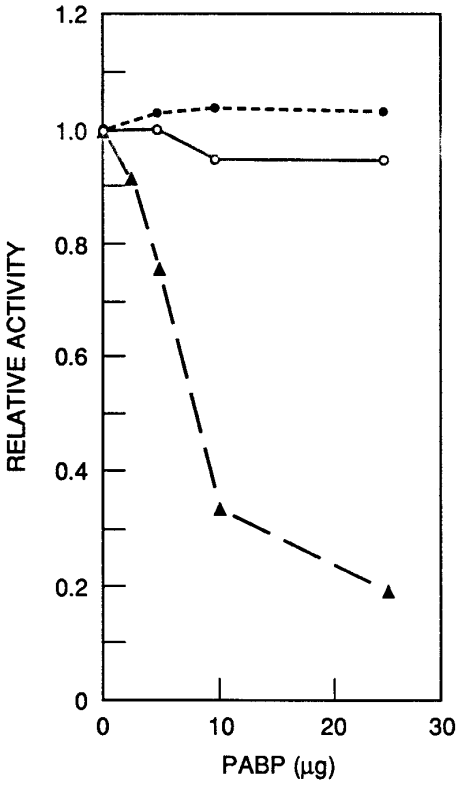

FIG. 5. Effect of PABP on poly(A) hydrolysis with XRN1 and HKE1. Reaction mixtures were set up and analyzed as described under Materials and Methods. The mixtures were incubated for 10 min on ice before the addition of XRN1 or HKE1. RNA was synthesized as described under Materials and Methods using a Promega control luciferase DNA as the template. XRN1 hydrolysis of poly(A) (triangles, dashed line); XRN1 hydrolysis of RNA (solid circles, dashed line); and HKE1 hydrolysis of poly(A) (open circles, solid line).

by GMP formation, was inhibited less than 25% as compared to the control RNA with both enzymes (results not shown).

Both exoribonucleases are active on a variety of deproteinized RNA substrates as well as ssDNA and dsDNA. The effect of proteins that form complexes with the polynucleotides on the rates of hydrolysis has not been analyzed. Such complexed proteins may shield the substrates, and such has been found to be the case with a human 3′ exoribonuclease as analyzed with poly(A) and PABP [31]. Fig. 5 shows an analysis of the effect of PABP on the hydrolysis of poly(A) by XRN1 and HKE1. XRN1 activity is inhibited by the protein with poly(A) as a substrate, but not with RNA. HKE1 hydrolysis of poly(A) is unaffected by PABP. It is possible that other complexed proteins may also block or stall XRN1 hydrolysis both in vitro and in vivo.

The results show that both 5′ exoribonucleases are strongly influenced by oligo(G)>9 tracts. These tracts stall the processive hydrolysis by the enzymes at the site of the tract insertion and the resulting 3′ RNA fragments are easily detected. The amount of accumulation of stall fragments is less with the RNA containing the stem-loop structures, but detectable by gel analysis. The overall influence of the stem-loops depends on their position in the RNA. These findings are relevant to both in vivo and in vitro studies that involve RNA modification for analysis of mRNA turnover pathways and RNA processing steps. Such investigations are being done in cells other than yeast. XRN1 and HKE1 are blocked very similarly by the two types of artificial secondary structures described here, suggesting that 5′ exoribonucleases from other eukaryotic cells may also be affected. The results show that a protein-polynucleotide complex may influence XRN1 activity. The PABP-poly(A) complex tested here did not affect HKE1 activity.

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