Multiple polyadenylation sites in a *Drosophila* tropomyosin gene are used to generate functional mRNAs

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ABSTRACT

The gene encoding muscle tropomyosin I in *Drosophila* is alternatively spliced in embryonic and thoracic muscle to generate two size classes of RNAs. By Northern blot analysis, the embryonic RNA class shows a broad RNA band of hybridization of 1.3 kb and a more sharply defined, less abundant RNA band at 1.6 kb. The thoracic class of RNAs, on the other hand, consists of a broad hybridization band at 1.7 kb and a more sharply defined band at 1.9 kb. Each size class of RNA encodes a different tropomyosin isoform. The two classes of alternatively spliced RNAs utilize the same 3' terminal exon of the gene. The DNA sequence of this exon reveals a cluster of several polyadenylation signals (AAUAAA) or polyadenylation-like signals. We show here by S1 nuclease protection analysis that at least five and possibly seven of these polyadenylation or polyadenylation-like sequences are associated with *in vivo* embryonic and thoracic mRNA cleavage processing sites. Six of these S1 sites are clustered within 119 bp and a seventh is located 255 bp downstream. At least one of the polyadenylation-like signal sequences appears to be an unusual AACAAA sequence. In addition we also show that these mRNAs function *in vitro* to synthesize muscle tropomyosins.

INTRODUCTION

The biogenesis of mature eukaryotic mRNA involves processing steps at the 5' and 3' ends and in the body of the primary transcript. These steps include the capping of the 5' end of the RNA, which occurs early during synthesis, and the splicing of introns or intervening sequences, if they exist (1-4). The 3' end of the RNA is processed by what now appears to be an endonucleolytic cleavage of the primary transcript (5-7). For most mRNAs this cleavage is closely coupled to the addition of a poly(A) sequence at the site of cleavage. A canonical AAUAAA is usually found preceding the site of polyadenylation in most eukaryotic mRNAs. Several studies have focused on this so-called polyadenylation (poly(A)) signal sequence and its possible role in cleavage and poly(A) addition. Mutations that delete or change the position of the poly(A) signal can inhibit cleavage and/or processing (8,9). In addition, sequences immediately following the cleavage site have also been

implicated in these events (6,7,10). Some genes contain more than one poly(A) signal sequence and cleavage site, and speculation has arisen as to the role of these multiple signals (11-17). For instance, they could serve as additional sequences of similar function to ensure complete termination or they could have some as yet unknown regulatory functions (18,19).

In our studies of the muscle tropomyosin I (mTm I) gene from Drosophila we have reported that the gene expresses a complex set of RNAs that fall into two classes. One class consists of a 1.3 and 1.6 kb set of RNAs that is expressed in late embryonic development and other tissue that have similar muscle (20). A second class of 1.7 and 1.9 kb RNAs is expressed in thorax and leg of the adult (21, Boardman and Storti, unpublished). On Northern blot analysis the 1.3 and the 1.7 kb RNAs migrate as a broad band suggesting heterogeneity in the length of these RNAs. The embryonic and thoracic classes of RNAs have been shown to be produced by alternative splicing of an internal exon in the primary transcript that gives rise to tropomyosin isoforms with different C-terminal 27 amino acids (21). One consequence of the alternative splicing patterns is that all four transcripts terminate in the same 3' terminal exon.

We have determined the DNA sequence of the 3' end of the mTm I gene and have identified a cluster of several poly(A) or poly(A)-like signals. We have determined and report here that at least five of these poly(A) signals are associated with sites of endonucleotic cleavage located within 119 bps of each other. This heterogeneity in termination accounts for the heterogeneity in size of the 1.3 kb embryonic and 1.7 kb thoracic RNAs. A seventh poly(A) signal and termination site is located 255 bp downstream from the cluster and it accounts for the 1.6 kb embryonic and 1.9 kb thoracic RNAs. We also demonstrate here that these mRNAs, fractionated by agarose gel electrophoresis, function in vitro to produced embryonic and thoracic muscle tropomyosin isoforms.

MATERIALS AND METHODS

RNA extraction

RNA from frozen embryos and thorax was prepared by the guanidinium isothiocyanate extraction method as described previously (21) and as modified from Chirgwin et al. (22). For Northern blot analysis the RNA (5 µg) was denatured and electrophoresed in 1.5% agarose containing 2.2 M formaldehyde for 5.5 hrs at 85V as described previously (21). The RNA was transferred to nitrocellulose according to Thomas (23) and hybridized with the pAS85-1

plasmid containing the mTm I gene. The hybridization conditions have been described (21).

RNA fractionation and in vitro translations

For cell-free translation of fractionated mRNA, 50 μ g of total embryonic or thoracic RNA was electrophoresed in 1% low-melt agarose gels containing methylmercury hydroxide as described by Miller (24). A single lane of RNA was sliced from the gel and stained with ethidium bromide to locate the RNA. The remaining lanes of the gel were sliced into 1 mm slices lengthwise in the region of the tropomyosin mRNAs (1.7-1.2 kb for Fig. 4 and 2.0-1.2 kb for Fig. 5) and the RNA extracted from the individual melted agarose slices as described (24). The final RNA pellets were suspended in water and translated in a cell-free protein-synthesizing system from rabbit reticulocyte lysates (25). The 35 S-methionine labelled products were separated by two-dimensional gel electrophoresis as described previously (25). The gels were fluorographed and exposed to X-ray film at -70° .

DNA sequence analysis

The DNA sequence of the 3' end of the gene was determined by the method of Maxam and Gilbert (26). The sequencing was performed in both directions and across all restriction sites.

S1 nuclease analysis

S₁ nuclease mapping was performed as described (27). $2-6 \times 10^5$ cpm (5-50 ng) of end-labeled single-stranded DNA fragments described in Fig. 3 were hybridized with approximately 60 μ g of total RNA isolated from frozen embryos (12-20h), thorax or whole adult flies, and yeast tRNA. Hybridizations were carried out in a final volume of 20 μ l in flame sealed, siliconized glass capillary tubes at 65°C for 15 h in 0.4 M NaCl, 40 mM PIPES (pH 6.4), 1 mM EDTA. The hybridization conditions were optimized for time and temperature of annealing and DNA concentration. Hybridization reactions were then injected into 20 volumes of ice cold S1 nuclease digestion buffer containing 20 μ g/ml denatured salmon sperm DNA and 500 U S1 nuclease. Digestions were carried out at 37°C for 60 min and terminated by ethanol precipitation. The digestion conditions were carefully controlled for enzyme concentration and time and temperature of digestion (Basi and Storti, described in detail elsewhere). The pellets were resuspended in formamide-dyes loading buffer and electrophoresed into 8% polyacrylamide, 8.3 M urea sequencing gels. The sizes of the S1 protected fragments were determined by comparison with 5' end labeled Hae III and Hinf I digested ϕ X 174 DNA size markers.

Figure 1. A. Northern blot analysis of total RNA from 12-20 hr embryos (E), thorax (T) and whole adult flies (A). The blot was hybridized with a nick-translated plasmid probe containing the mTm I gene (20,21). B. Schematic diagram of the mTm I gene and the alternative splicing pathway encoding the embryonic and thoracic RNAs. For details consult Basi et al. (21).

RESULTS

The muscle tropomyosin gene (mTm I) is developmentally expressed during embryogenesis. During mid-embryonic development (9-11 hr) transcripts first begin to accumulate to detectable levels and continues to accumulate throughout embryonic and larval development (20, Boardman and Storti unpublished). Two bands of RNA can be detected by Northern blot analysis of embryonic RNA when hybridized with an mTm I gene probe. A broad band of hybridization occurs having a mean size of 1.3 kb and a more sharply defined less abundant band at 1.6 kb (Fig. 1A, lane E). During pupal development a switch in expression occurs such that in thorax the major transcripts are now 1.7 and 1.9 kb. We have shown that the embryonic and thoracic transcripts arise by differential splicing of exons encoding the C-terminal 27 amino acids of the tropomyosin protein. Figure 1B shows a schematic diagram of the gene and the pathway for splicing of the embryonic and thoracic RNAs. It can be seen that both classes of RNA utilize the same exon (fifth exon of the gene) to encode the 3' end of the RNA. The additional 429 bp internal exon of the thoracic RNA accounts for the difference in size between the 1.3 kb embryonic and 1.7 kb thoracic RNAs as measured by formaldehyde gel electrophoresis. The larger embryonic and thoracic RNA differ in size by approximately the same amount.

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atttccaaccaccatttgcactccgcag  AC GAG TTG GGC ATC AAC AAG GAC AGG TAC AAG TCC CTG GCC GAC GAG ATG GAC
    Asp Glu Leu Gly Ile Asn Lys Asp Arg Tyr Lys Ser Leu Ala Asp Glu Met Asp
1
TCC ACA TTC GCC GAA TTG GCT GGC TAC TAA GTTGGCCACCAGTCCCATTATACCAATCCACACCAGCGGAGGAAGATGGAGACGAC
Ser Thr Phe Ala Glu Leu Ala Gly Tyr *
90
GACGAGATGGGAACTCTACTAATTGTAAACGAATCTCAAAATTGATTGGCCAACACGAAGATCGGGCGCCGAGATACCAAGAACAACACCATTTGT
190
GTATGCAATTCCTTTATAAATCTATATATTTTCATAATAAATAAATAACAACACCAACAGATCAACAACAGCAGCAAGGCCAACGAGCATAGAAAGCAAT
290
GGAAATTCCTTTGTTTTATAAAAAATAAATATCTAAAGTCAAAAGTAGCAGAAATGTTATAAGTAAACAAAACGAAAACAAGAACACGAATTTTGTAAT
390
TTTAAAGTCAATGTTTAAAAATATTAAAGAGCGCTTGCAGTCAGGCAATAAGAGCGAGAGAGCTAGAGAGAGTGCCCGAGAAAGAGACGGGGCTAAATTT
490
AAAAATTTAAGATCGCGCTGCGCTGCTTTACTTTTCAGTTTATATCTTACAAATTACACCACAAACAAACAAAAAACACCCACAAAAAAGTATAAAAAAT
590
TTATAAAACCTTGTCTAAAAAAATATATGTTgttgatgttggcttcaatgatgcacaaaccgaattctttgccccttcagogaatcgcgctgaagtt
690

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Figure 2. DNA sequence of exon 5 (see Fig. 1B) of the mTm I gene encoding the 3' end of the mRNA. The numbering of nucleotides begins at the Hinf I site as shown in Fig. 3A and described in the text. The approximate location of the S1 nuclease cleavage sites are indicated by arrows. The error is ± 5 bp. The poly(A) signal sequences are underlined.

3' end analysis

We have determined the DNA sequence of the fifth exon that encodes the 3' end of the gene. It is shown in Figure 2. The numbering of nucleotides is from the 5' Hinf I site used in the S1 nuclease experiments described below. The first occurrence of a consensus poly(A) signal (AAUAAA) is at position 224 of the sequence. This is followed by an overlapping poly(A) sequence at position 228. A third site occurs at position 314 in the sequence. We have determined the 3' end of the embryonic and thoracic mRNAs by S1 nuclease analysis. Figure 3A shows a partial schematic restriction map of the 3' end of the gene and the strategy for mapping the ends of the transcripts. A 677 nt single stranded Hinf I probe, 3' end labelled at the Hinf I site, was used to map the 3' end of the RNAs. The probe was hybridized with equal quantities of embryo and adult (thorax) RNA, digested with S1 nuclease and the products separated by electrophoresis. Adult RNA was used instead of thorax RNA because it was more readily available in large quantities. The distribution of tropomyosin RNAs is the same in whole adult RNA as it is in RNA from thorax (Fig. 1A). The results of this experiment are displayed in Fig. 3B and C. Lane C is a different experiment from lane B where the S1 digestion products have been electrophoresed further into the gel. The nuclease digestion of this probe after hybridization with both embryo and adult RNAs reveals a series of five strong bands common to both embryo and adult RNA protected samples (Fig. 3B). The smallest protected fragment is about 250 nt in size,

Figure 3. A. Schematic diagram showing a partial restriction and gene structure map indicating the 3' end labelled single stranded probe used to S1 map the 3' end of the RNAs. Boxed areas represents exons, thin line represents intron and 3' unmapped region of the gene. The arrow denotes the probe used in the experiment shown in B and C. The end labelled site is indicated by an asterisk. B and C are autoradiograms of gels from similar experiments showing S1 nuclease protected fragments electrophoresed for 2 hrs (B) and 4 hrs (C) at 1700 V. E and A refer to embryonic and adult RNA protected fragments. The arrows in B correspond to the size bands in C. The size of the fragments was determined by the position of 5' end labeled ϕ X 174 Hae III and Hinf I digested size markers. The exposure was 8 hrs.

and upon longer electrophoresis can be resolved as a 246 and 251 nt doublet (Fig. 3C). We estimate an error of ± 5 bp in our sizing of the nuclease protected bands, and hence, approximate the site of cleavage for polyadenylation by a similar amount to the region around the arrows in Fig.

2. Examination of the DNA sequence shows that the sequence AATAAATAAA occurs at position 224, approximately 22 bp upstream from the first sites of S1 cleavage. This sequence corresponds to an overlapping poly(A) signal and as revealed by the doublet S1 protected bands, indicates that both signals are functional in directing poly(A) addition and/or processing at the two S1 cleavage sites downstream.

The third and fourth highest protected fragments are approximately 288 and 365 nts in size, and as can be seen, the 288 band nt may be slightly more pronounced in the lane in which the probe was hybridized with adult (thorax) RNA as compared with embryo RNA. However, although equal amounts of RNA were used in these experiments, we cannot be completely certain of the significance of this difference in intensity. The DNA sequence in this region of the gene does not reveal an AATAAA poly(A) signal but instead there is an AACAAA sequence at position 262 and an ATAAA sequence approximately 26 and 16 nucleotides upstream from the S1 cleavage site, respectively. The only difference between the two sequences and the consensus AATAAA is the substitution of a C for a T, (both pyrimidines) in the former, and the absence of an A in the latter. This result indicates that these sequences can also be utilized as a polyadenylation and processing sites.

The next prominent higher mw-protected band is approximately 620 nts in size; the DNA sequence of the 620 nt is preceeded by two ATAAA poly(A)-like signals, the closest at position 593. Finally, we consistently observe two weak nuclease protected bands at 262 (more apparent in B) and 345 nts in size and interpret these as likely representing a sixth and seventh cleavage and termination site. The DNA sequence preceding these map points at positions 241 and 314 reveal a AACAAA sequence and a consensus poly(A) signal, AATAAA. It is also possible, however, that the 262 nt protected fragment arises from polyadenylation at that site directed by either, or both of the overlapping signals at position 229. The faint band below the 288 nt band in C has only been observed in this experiment.

The lower cluster of nuclease protected bands, which map within 119 bp of one another, therefore, map at least four and probably six discrete 3'termini in the mTm I mRNAs. The 1.3 kb embryo and 1.7 kb thoracic transcripts which are observed as rather broad bands on northern blots, can thus be explained on

the basis of these results as arising from polyadenylation of the transcripts after the six clustered poly(A) sites downstream in the gene. Finally, the 1.6 kb embryo and the 1.9 kb thorax transcripts, which by contrast have always appeared as single discrete bands, can be explained by arising from polyadenylation of the primary transcript at the farthest poly(A) site located an additional 255 nts downstream in the gene. Furthermore, the close agreement between the sizes of the transcripts as determined by Northern blot analysis and by adding up the sizes of the exons determined by S1 nuclease analysis and primer extension analysis (see ref. 21) indicates that the poly(A) tail length of the mRNAs cannot be longer than 50-100 residues.

Separation and in vitro translation of embryonic and thoracic RNAs.

The functional significance of the different size mRNAs was tested by fractionating the RNAs by electrophoresis in gels containing methylmercury hydroxide and by translating *in vitro* the fractionated RNAs (24). Although it is not possible to resolve the individual RNAs that make up the 1.3 kb and the 1.7 kb RNA bands, both RNA bands are easily separable from the 1.6 and 1.9 kb RNAs (Figure 1). Accordingly, embryonic RNAs were fractionated by denaturing gel electrophoresis. The gel was sliced into 1 mm slices in the region of the tropomyosin mRNAs, and the RNA was extracted from each slice and translated in a cell-free translation system from rabbit-reticulocyte lysates. The ³⁵S-methionine translation products of RNA from individual gel slices were electrophoresed in two-dimensional gels. The 1.3 and 1.6 kb set of RNAs have been shown previously to hybrid-select and translate the mTm I embryonic isoform (20). The 1.7 and 1.9 kb RNAs encode the thoracic isoform (21). Both tropomyosin isoforms can be distinguished in two-dimensional gels. In this experiment the actin and muscle tropomyosin II isoform (mTm II) serve as internal markers to roughly correlate the translation products with the size of the mRNAs. The former is encoded by several mRNAs that range in size from 1.6-2.0 kb. The mTm II mRNA is 1.4 kb in size.

Representative results of the embryonic RNA analysis are shown in Figure 4. Only translations from gel slices corresponding to the different size tropomyosin RNAs are shown. Panel 4 shows the translation products from the 1.6 kb region of the RNA gel. It can be seen that the only tropomyosin expressed in this region of the gel is the mTm I embryonic isoform. The slight trailing of the spot in the direction towards the acid side of the gel occurs with older stocks of urea sample buffer. Note the presence of actin and the abundant protein above actin at about 60,000 daltons. Also note the absence of mTm II protein. Panels 6 and 8 are slices of the gel between the

Figure 4. Fluorogram of ^{35}S -methionine labelled embryo RNA cell-free translation products electrophoresed in two-dimensional gels. The embryo RNA gel was cut into 20 1 mm fractions. The area of the gel cut spanned the 1.7-1.2 kb region of the gel. The numbers of each gel refer to the gel slice numbered from the top to bottom direction of the gel. I and II refer the mTm I and mTm II isoforms; E is an endogenous product synthesized when no RNA is added to the translation; A are the actin isoforms. The exposure of all samples was 3 days.

1.3 and 1.6 kb RNAs. The amounts of actin and the mTm I protein are now reduced in fraction 6. In fraction 8 actin is barely detectable. The 60,000 dalton protein is also diminished. In fraction 8 the mTm II protein is beginning to appear and serves to indicate the 1.4 kb size range of the gel. In Panel 10 there are much greater quantities of the mTm I and mTm II

Figure 5. Fluorogram of ^{35}S -methionine labelled adult RNA cell-free translation products electrophoresed in two-dimensional gels. The thorax RNA gel was cut into 28 1 mm slices. The area of the gel slices spanned the 2.0-1.2 kb region of the gel. The number of each gel refers to the gel slice number from the top to the bottom of the gel. I and II refer to the mTm I and mTm II isoforms; T refers to the mTm I thorax isoform; E is the endogenous product; A is actin. The exposure for 4 and 10 was 14 days. The exposure for 18 and 26 was for 7 days.

isoforms, reflecting the large amounts of the respective mRNAs in this region of the gel. These results clearly show that both the 1.3 and 1.6 kb RNAs synthesize the mTm I tropomyosin isoform as expected if these RNAs are functional mRNAs.

A similar analysis using RNA from whole adults as the source for thorax

RNA is presented in Figure 5. The difference in two-dimensional gel mobility between the embryonic and thoracic mTm I isoforms can be seen relative to the position of the mTm II. The 1.3 and 1.6 kb embryonic mTm I transcripts make up about 10-20% of the mTm I RNA in thorax. Thus, small amounts of mTm I embryonic isoform are expected in the translations. The panels show translations corresponding to gel slices from the 1.9 to the 1.3 kb region of the RNA gel. Gel fraction 4 from the 1.9 kb region of the gel contains RNA that translates the thoracic mTm I. The actin has smeared in the gels shown here. Fraction 10, which corresponds to the 1.7 kb region of the gel, shows larger quantities of the thoracic mTm I isoform, owing to the large amounts of the 1.7 kb compared to the 1.9 kb RNA. Also note the appearance of small amounts of the mTm I embryonic isoform encoded by the 1.6 kb RNA. Fraction 26 from the 1.3 kb region of the gel shows the continued translation of the mTm I embryonic isoform and the synthesis of the mTm II protein. The mTm I thoracic isoform, on the other hand, is not translated by mRNAs in this size range. These results show a direct correlation in the distribution of tropomyosin isoforms and the position of their respective size fractionated mRNAs in the gel. Thus, the 1.9 and 1.6 kb thorax RNAs both function in vitro and translate the thoracic mTm I isoform. We cannot separate the individual mRNAs in the 1.3 and 1.7 kb region of the gel that correspond to the clustered poly(A) sites, but the broadness in the distribution of the mTm I translation products correlates with the sizes of the RNAs. We have not attempted to accurately quantitate the amount of tropomyosin translated in each sample because the efficiency of translation can be effected by the extraction from the gel and the difference in size of the mRNAs. It appears from observing the gels, however, that the amount of tropomyosin correlates closely with the relative amount of the RNAs as determined by RNA blot analysis (Fig. 1). This would indicate that the different polyadenylated mRNAs are translated with approximately equal efficiency.

DISCUSSION

The results presented here show that the mTm I gene from Drosophila utilizes at least six, possibly seven poly(A) signals in vivo in both embryonic and thoracic RNAs. Three of the signals are the more common AATAAA sequence. Two are the ATAAA sequence and two are the hitherto unreported AACAAA. The AATAAA signal occurs three times in this region of the gene, and is used all three times, although one is used in greatly reduced amounts. There are multiple occurrences of the ATAAA sequence, however not each

occurrence is utilized. The sequence ATAAA is found by itself, i.e. excluding the times when it is observed in the sequence AATAAA, three other times in addition to the two times when it is used for polyadenylation. The first two occurrences where it is not used are at sequence positions 205 and 308. Examination of the DNA sequence in these regions shows the presence of the more commonly used AATAAA signals shortly downstream, and hence they may serve as stronger poly(A) signals than ATAAA. It is also possible that we are observing the effect of the two type sequences acting in concert. The third occurrence where this signal does not appear to be used is at sequence position 581. In this instance the sequence is actually found to occur twice within a 12 bp distance, the second one being at sequence position 593. In this case it is entirely possible that both are being used and their detection is precluded by the size of the nuclease protected fragment which results (610 nt) from the probe we employed in this experiment. Such small differences in sizes of a nuclease-protected fragment this large would not have been detected under the electrophoretic conditions we employed in this experiment. The poly(A) signal AACAAA is found to occur a total of five times in the 3' untranslated region of the gene. However, as noted earlier, it is used only twice, and in this instance they are found within 21 bp of each other and nestled between two AATAAA polyadenylation signals, the more distal of the two is used with greater frequency. In addition, we cannot rule out the possibility that other sequences in this region also effect the site of cleavage and polyadenylation.

The unusual polyadenylation signals observed in the mTm I gene, ATAAA and AACAAA, are not the only reported deviations from the more common AATAAA signal. The sequence ATTAAA is a functional polyadenylation signal in the chicken lysozyme gene (28) as well as in the mouse pancreas α -amylase gene (12). In addition, the sequence AATATA is also functional in the mouse α -amylase gene (13). With respect to the AACAAA polyadenylation site, Montell et al. (9) have recently reported on the efficacy of a U $\rightarrow$ G transversion mutation in the hexanucleotide sequence. They found that the mutant was not cleaved as efficiently as the wild type, however, the mutation did not affect the specificity of cleavage nor polyadenylation at the site of cleavage. In our case, we observe a naturally occurring U $\rightarrow$ C transition mutation in the hexanucleotide sequence which appears to be utilized with higher efficiency in a tissue specific fashion in the one out of two times it is used.

It has been proposed recently that U4 small nuclear ribonuclear proteins may be implicated in the polyadenylation mechanism and that the secondary

sequence element CAYUG, located either immediately upstream or downstream of the primary AAUAAA sequence element may be related to cleavage site selection (5). Survey of the mTm I gene sequence in the regions of the seven poly(A) signals does not show any strong match with this consensus pentanucleotide sequence.

Multiple polyadenylation sites have been found in many eukaryotic genes (11-17). In certain instances, polyadenylation at a given site has been correlated with the tissue-specific expression of that gene, whereas in other cases, no such correlation has been observed (19). We have analyzed the expression of the mTm I gene in embryos during larval development and in different adult tissue (20, Boardman and Storti, unpublished). In most tissue examined the relative amounts of the 1.3-1.6 kb RNAs and the 1.7-1.9 kb RNAs remain constant. We have found, however, that in third instar larvae there appears to be a significant decrease (20% to 5%) in the amount of the 1.6 kb RNA relative to the 1.3 kb RNA, indicating that the last poly(A) site at position 593 may not be utilized in this tissue. This would suggest some developmental specificity to the site selection process, however, this has not been investigated further. Another possibility for multiple poly(A) sites might be to insure more efficient 3' processing (cleavage) and polyadenylation. This may be particularly important for genes, such as tropomyosin, that are transcribed at high levels and/or have weak processing signals. The fact that most of the poly(A) sites are used with approximate equal frequency indicates that site selection or processing is not sequential. If it were sequential, we might expect that each successive poly(A) site would be associated with fewer termination events. Our data, however, indicate that those poly(A) sites that are used are done so with approximately equal frequency.

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