Electrophoretic patterns of deadenylylated chorion and globin mRNAs

[RNase H/insect mRNA/poly(A)/mRNA resolution]

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ABSTRACT   Treatment of silkmoth chorion mRNAs with calf thymus RNase H (EC 3.1.4.34; RNA-DNA-hybrid ribonucleotidohydrolase) in the presence of oligo(dT) specifically and effectively removes the 3'-terminal poly(A) sequences. Excision of non-poly(A) fragments cannot be detected. Under these conditions, RNase H leads to increased electrophoretic homogeneity of rabbit globin mRNA, presumably as a result of removal of poly(A) sequences that are inherently variable in length. Treatment with RNase H converts the three diffuse zones of messages for the several chorion proteins into multiple sharp bands.

It is commonly accepted that fractionation of mRNAs is inferior to protein fractionation. We have encountered this problem in our studies on differentiation in the follicular cells of silkworms (1–4). At the end of oogenesis, these cells are highly specialized for programmed sequential production of a number of related proteins, which are secreted extracellularly to form a complex protective eggshell or chorion around the oocyte. Resolution of the proteins on sodium dodecyl sulfate (NaDodSO₄)/polyacrylamide gels (1–3, 21) reveals a minimum of 15 low molecular weight components in three size classes (A, B, and C, approximate average molecular weights of 9,000, 13,000, and 18,000, respectively); further heterogeneity is evident on isoelectric focusing gels (2). Isolation of chorion mRNAs was accomplished with relative ease (2–4, 13) because of certain features of this system [>95% specialization for chorion protein synthesis; conveniently small size of the proteins and of the mRNAs; presence of sufficiently long poly(A) sequences to permit purification through binding to oligo(dT)-cellulose]. However, in contrast to the resolution of numerous chorion proteins, only three diffuse zones of chorion mRNAs (2, 3, 13) were resolved by common procedures (zones 1, 2, and 3, possibly corresponding to protein classes A, B, and C, respectively).

The resolution of chorion mRNAs seemed considerably lower than that attained with histone mRNAs, which lack poly(A) (25). We reasoned that the presence of poly(A) sequences, which are known to be polydisperse in size (13), might broaden the size distribution for individual mRNA species to a degree sufficient for overlap between species to occur. This might create a single diffuse zone out of what would otherwise be a series of distinct mRNA bands. Here we report that specific removal of the poly(A) by calf thymus RNase H indeed leads to sharpening of the electrophoretic band for a model mRNA, globin mRNA. In the case of chorion messages, deadenylation leads to the formation of distinct electrophoretic bands, nearly as numerous as the protein components that can be resolved by NaDodSO₄/polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Animals. Chorionating follicles were dissected from commercially obtained *Antheraea polyphemus* and *Antheraea*

Abbreviations: PEI, polyethyleneimine; NT, nucleotides; NaDodSO₄, sodium dodecyl sulfate.

pernyi as described (1, 2).

Radioactive Labeling and Purification of Chorion mRNAs. Chorion mRNAs were purified and labeled in 6- to 8-hr organ culture as described (3, 4, 5). The mRNA was recovered from Mg⁺²-precipitated polysomes and was bound to oligo(dT)-cellulose at least once. ¹²⁵I-Labeled chorion or rabbit globin mRNAs were prepared as described (5). All RNA preparations not exposed to RNase H are called intact.

RNase H Digestion of mRNA. Homogeneous calf thymus RNase H (RNA-DNA-hybrid ribonucleotidohydrolase; EC 3.1.4.34) was obtained from Dr. J. G. Stavrianopoulos (see ref. 7 for storage buffers, digestion conditions, etc.). Digestions were performed for up to 45 min at 35°, and were monitored by precipitation of the undigested material with cold trichloroacetic acid.

For mRNA digestions (7), a minimum ratio of 1 µg of enzyme:5 µg of mRNA was maintained. The mRNA was preincubated at room temperature, in water and then in 0.05 M KCl, for 10 min each, with oligo(dT) [Collaborative Research; d(pT)₁₂₋₁₈]; 10 µg of RNA:1 µg of oligo(dT)].

Recovery of Deadenylylated mRNA and of Low Molecular Weight Products of RNase H Digestion. After addition of 1, 0.2, and 0.2 volume of 0.05 M KCl, 0.2 M EDTA, and glycerol, respectively, reaction mixtures were passed through a freshly made Sephadex G-150 plus nitrocellulose column assembly (7). The elution buffer was 0.1 M KCl. Low molecular weight digestion products and the deadenylated RNA were recovered after passage through the nitrocellulose part of the column assembly (back and front peaks, respectively).

RNA Electrophoresis. As indicated, 6%, 8.5%, or 12% polyacrylamide slab gels containing 7 M urea were used (3, 4). Labeled RNA was detected by autoradiography. Using the autoradiogram as a guide, bands were sometimes excised and the RNA was eluted (4, 8).

Nucleotide Base Compositions. RNA of oligonucleotide samples was lyophilized to dryness and hydrolyzed in 20 µl of 0.3 M KOH at 95° for 1–2 hr, under mineral oil. The products were spotted on Whatman 540 paper and analyzed by electrophoresis (9).

Polynucleotide Phosphorylase Digestion of RNA. Samples of RNA (<1 µg) dissolved in 10 or 20 µl of water were digested with *Micrococcus luteus* polynucleotide phosphorylase (EC 2.7.7.8; Worthington), by mixing with an equal volume of 2× buffer (0.10 M Tris·HCl, pH 7.5, 0.03 M MgCl₂, 0.03 M potassium phosphate) and with 0.5 volume of enzyme solution (5 mg/ml in 1× of the above buffer).

Analysis of Polynucleotide Phosphorylase Digestion Products. Nucleotide diphosphates were fractionated on 20 × 20 cm polyethyleneimine (PEI)-cellulose sheets (Brinkman Instruments, predeveloped with water and dried). Small aliquots (4–8 µl) of total reaction mixtures were spotted and dried, and the chromatogram was developed with 0.4 M KH₂PO₄ adjusted to pH 3.5 with phosphoric acid. Radioactive spots (including the undigested material at the ori-

Table 1. Extent of RNase H digestion of silkmoth chorion mRNA*

RNA type	Time of digestion (min)	Acid-precipitable cpm†	% Digested‡
polyphemus [³²P]mRNA			
Experiment 1ᵃ	0	764 ± 16	—
	15	641 ± 12	16
	30	642 ± 3	16
	45	640 ± 3	16
Experiment 2ᵇ	0	416 ± 8	—
	5	313 ± 9	13
	10	312 ± 7	13
	30	314 ± 0	13
pernyi [³²P]mRNA			
Experiment 1ᶜ	0	699 ± 9	—
	25	639 ± 8	9
Experiment 2ᵇ	0	7,330 ± 150	—
	25	6,320 ± 210	14
pernyi ¹²⁵ I-labeled mRNAᵈ	0	22,470 ± 690	—
	45	23,160 ± 790	0

* Unless stated otherwise, the RNA was obtained from Mg^{+2}-precipitated polysomes of follicles labeled with [³²P]phosphate in culture for 6 hr.

† Duplicate aliquots of the incubation mixture were assayed by trichloroacetic acid precipitation at each time point. Counting time was usually 50 min, and in any case sufficient to reduce counting error below 1.5%.

‡ Percentages were calculated from the decrease of trichloroacetic acid-precipitable cpm, relative to the zero time value for each experiment.

ᵃ The mRNA was additionally fractionated on a 6% polyacrylamide gel (Figs. 3 and 5), and zone 2 was recovered and used in this experiment.

ᵇ The mRNA was additionally purified by a second oligo(dT)-cellulose binding step.

ᶜ The mRNA was obtained from an 8-hr labeling experiment and was additionally purified by sucrose gradient centrifugation and a second oligo(dT)-cellulose binding step.

ᵈ Unlabeled mRNA purified as for ᶜ was radioiodinated and was then repurified by a third oligo(dT)-cellulose binding step.

gin) were located by autoradiography and quantitated by liquid scintillation.

RESULTS

Extent of Chorion mRNA Digestion by RNase H. Table 1 documents the extent of digestion of chorion mRNAs by RNase H, in the presence of oligo(dT). Preparations of ³²P-labeled chorion mRNA were exposed to the enzyme, and the decrease in trichloroacetic acid-precipitable radioactivity was monitored as a function of time. We conclude that approximately 14% of the nucleotides can be removed from these mRNA preparations by RNase H digestion. The reaction goes to completion rapidly (5 to 15 min).

Table 1 also includes the results of an experiment in which iodinated chorion mRNAs were exposed to RNase H. In this case, no radioactivity was made acid-soluble. Iodine is known to label almost exclusively the cytosine bases of RNA (10; but see ref. 11). Thus, this experiment is important in suggesting that no cytidine-containing oligonucleotides are produced in the course of the reaction—in agreement with the expectation that only poly(A) is digested under our conditions.

The Base Composition of Oligonucleotides Produced by RNase H. Those products of RNase H digestion that were
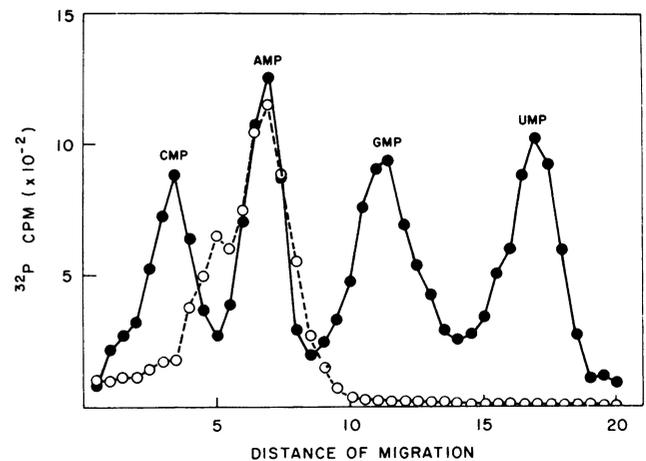
FIG. 1. Base composition of oligonucleotides produced by RNase H digestion of ³²P-labeled *polyphemus* mRNA. ³²P-Labeled rRNA was processed in parallel as a control. (●) rRNA, 21,660 total cpm (plotted at a 1.4× expanded scale for optimal display). (○) RNase H digestion products, 12,150 total cpm.

retarded by a Sephadex G-150 column were collected, alkali-digested, and analyzed by high voltage paper electrophoresis for determination of base composition. RNase H digestion of poly(dT)·poly([³²P] rA) hybrids releases oligomers of adenylic acid containing between 2 and 10 bases (12). In several experiments, the low molecular weight products of chorion mRNA (from either moth species) contained virtually only adenylic acid (Fig. 1). GMP and UMP were undetectable (detection limit 2% of AMP). The absence of CMP could not be established from these experiments at a comparable level of sensitivity, because AMP dimers (13) and oligomers overlap the CMP spot; however, the absence of cytidine-containing oligonucleotides was demonstrated in Table 1. We conclude that only the poly(A) portion of chorion mRNA molecules is degraded by RNase H.

The 3′ End of Intact and RNase H-Digested Chorion mRNA. The 3′ exonucleolytic activity of polynucleotide phosphorylase can be used to probe the composition of the 3′ end of an RNA molecule (14, 15). Beginning at a 3′-OH terminus, the enzyme digests in a 3′ → 5′ direction in a processive manner, producing nucleotide diphosphates (15). The products can be identified and quantified by PEI-cellulose ascending thin-layer chromatography.

Figure 2A and B confirms that the 3′ end of intact chorion mRNA from either A. *pernyi* or A. *polyphemus* is poly(A) (13). Virtually the only nucleotide released by the conveniently slow enzymatic reaction at 5° is ADP. Surprisingly, the reaction with A. *polyphemus* mRNA appears to plateau when the ADP produced amounts to only 1% of the total RNA (two experiments); with A. *pernyi* mRNA, the ADP produced in 30 min amounts to as much as 12% of the total (two experiments), in agreement with the expectation from Table 1. The reason for this difference is not known. In the case of rabbit globin mRNA, digestion at low temperature and high salt does not proceed beyond the poly(A) sequence (15), possibly because of interfering secondary structure (16). It remains to be established whether a chemical or a structural difference between the 3′ ends of chorion mRNAs of A. *polyphemus* and A. *pernyi* accounts for their differing sensitivities to polynucleotide phosphorylase at 5°.

Preliminary, low radioactivity experiments suggested that RNase H-digested chorion mRNAs from either species may be resistant to polynucleotide phosphorylase at 5° under our conditions. This resistance could not be due to the absence of a 3′-OH (12). To circumvent this resistance, RNase H-di-

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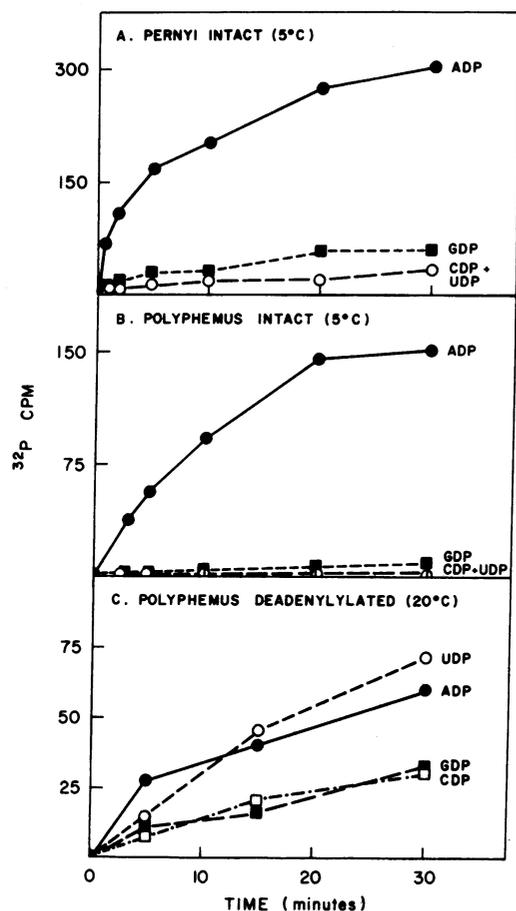
FIG. 2. The composition of the 3' ends of intact (A and B) and RNase H-digested (C) chorion mRNAs, as revealed by the nucleotide diphosphates released by polynucleotide phosphorylase. Data are normalized to equal aliquot volumes. (A) Intact *A. pernyi* [^{32}P]mRNA, purified as in Table 1, footnote [c]. Reaction at 5°. Total radioactivity per aliquot, 2,663 to 2,983 cpm. (B) Intact *A. polyphemus* [^{32}P]mRNA, purified as in Table 1, footnote [b]. Reaction at 5°. Total radioactivity per aliquot, 14,890 to 17,920 cpm. (C) RNase H-digested *A. polyphemus* [^{32}P]mRNA, purified as in Table 1, footnote [b]. Reaction at 20°. Total radioactivity per aliquot, 1,176 to 1,272 cpm. Counting conditions as in Table 1.

gested *polyphemus* chorion mRNA was exposed to polynucleotide phosphorylase at 20°[‡] (Fig. 2C). All four nucleotide diphosphates were released progressively, without a detectable time lag. The products appeared to be slightly enriched for ADP (45% of total NDP) at 5 min of digestion, when 4.7% of the RNA had been degraded. From these results, given that the non-poly(A) part of chorion mRNA is about 30% adenylic acid (2, 13) and that the average size of RNase H-treated chorion mRNA is about 580 nucleotides (see below), it can be calculated that, on the average, no more than 4 adenylic acid residues remain from the 3' poly(A) after RNase H digestion. It should be noted that the 3' ends of deadenylylated chorion mRNAs appear to be deficient in - G+C relative to the total mRNA composition (2, 13). The sequenced 3' ends of globin and immunoglobin mRNA are similarly deficient in G+C (16).

[‡] In a preliminary experiment at 20°, the release by polynucleotide phosphorylase of ^{125}I-labeled cytidine diphosphate from intact iodinated *A. pernyi* mRNA was delayed, relative to the release of [^{32}P]cytidine diphosphate from RNase H-digested *A. polyphemus* mRNA (2.1% and 8.1% of the total labeled cytidine, respectively, at 15 min). No labeled nucleotide other than ^{125}I-labeled cytidine diphosphate was released from the iodinated message.
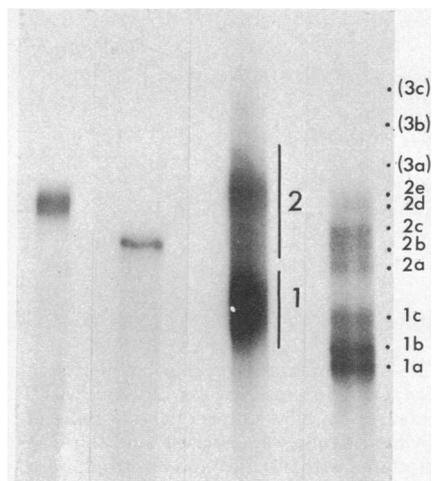
FIG. 3. Autoradiogram of electrophoretically analyzed, intact and RNase H-digested mRNAs. Electrophoresis was in an 8.5% polyacrylamide slab gel (36 hr at 320 volts). From left to right: Slot 1, intact globin mRNA; slot 2, digested globin mRNA; slot 3, intact chorion mRNA (zones 1 and 2 are indicated); slot 4, digested chorion mRNA (resolved components are indicated, in parentheses, if detectable only on the original autoradiogram). In this and each of the subsequent figures, all slots are from a single slab gel; different exposures have been used to compensate for different amounts of radioactivity in individual slots.

Electrophoretic Analysis of RNase H-Digested ^{125}I-Labeled mRNAs. In order to study the effects of RNase H digestion on mRNA size, extensively purified rabbit globin mRNA and *A. pernyi* chorion mRNA were labeled with ^{125}I and analyzed by electrophoresis on an 8.5% polyacrylamide slab gel, with and without prior exposure to RNase H. The labeled RNAs were detected by autoradiography (Fig. 3). Approximate size estimates of the digested species could be obtained by reference to the known sizes of the intact mRNAs (5, 17), assuming that mobility is linearly related to the logarithm of length, in nucleotides (NT).

Intact globin mRNA (assumed average size, 670 NT) migrated as a broad zone. The breadth of the zone corresponded to an approximate range of ±30 NT around the mean. This range is consistent with the known size difference of intact α and β globin mRNA (17) and the polydisperse nature of the poly(A) in total globin mRNA (18). After digestion with RNase H, the average size was reduced by approximately 60 NT, and the mRNA migrated as a much tighter band. Surprisingly, one rather than two bands was detected; unless our globin mRNA preparation is highly enriched for one of the two message species, the results suggest that the well-known size difference of α and β globin messages (17) may be due to a difference in the average size of the respective poly(A) sequences (see also ref. 19).

Intact chorion mRNA consists of two major, diffuse zones, called zones 1 and 2 in the order of increasing molecular weight (3, 13). The midpoint of zone 2 nearly comigrates with globin mRNA (3), whereas zone 1 is smaller by approximately 150 NT (5). A third, very minor, higher molecular weight component exists (zone 3), but is difficult to detect except at early developmental stages, when the minor, relatively high molecular weight chorion proteins (class C) are being synthesized (2, 20). The electropherogram of Fig. 3 is typical of intact zones 1 and 2 (2, 3, 13, 20).

After exposure to RNase H, instead of broad zones, a number of distinct bands became apparent (Fig. 3). The overall size distribution was shifted downwards by approximately 30 to 40 NT, which corresponds to the known size of
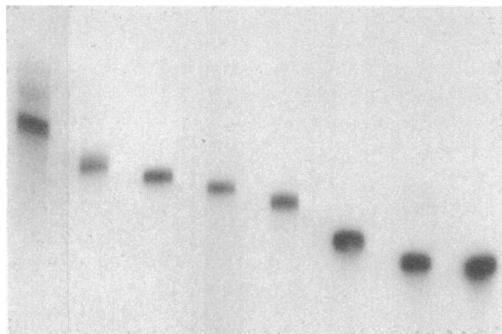
FIG. 4. Re-electrophoresis of chorion mRNA bands. ^{125}I-Labeled *A. pernyi* chorion mRNA was digested with RNase H and electrophoresed on an 8.5% polyacrylamide slab gel (as in Fig. 3, slot 4). Individual bands were excised, and the RNA of each was eluted, precipitated with ethanol, and electrophoresed without further purification, in the indicated slots of a second, identical gel. From left to right: Slot 1, components 3c, 3b, and 3a; slot 2, components 2e and 2d; slots 3 to 8, components 2c, 2b, 2a, 1c, 1b, and 1a, respectively.

FIG. 5. Electrophoresis of ^{32}P-labeled chorion mRNAs. *A. pernyi* and *A. polyphemus* mRNAs were electrophoresed on a 6% polyacrylamide gel after the first oligo(dT)-cellulose purification step, and zones 1 and 2 were recovered separately. The mRNAs were further purified by again binding to oligo(dT)-cellulose (this step also removed gel impurities; ref. 4). Portions of each mRNA sample were subjected to RNase H digestion and recovered as described in *Materials and Methods*. Intact and digested samples were analyzed on a 6% polyacrylamide gel (24 hr at 320 volts). From left to right: Slots 1–4 have *A. pernyi* samples; resolved bands are marked with dots, and the components labeled in Fig. 3 are indicated. Slot 1, digested zone 1; slot 2, digested zone 2; slot 3, digested zones 1 and 2, combined; slot 4, intact zones 1 and 2, combined. Slots 5–8 have *A. polyphemus* samples; individual bands are indicated by dots. Slot 5, intact zone 2; slot 6, intact zone 1; slot 7, digested zone 2; slot 8, digested zone 1.

poly(A) in long-term labeled chorion mRNA [briefly labeled chorion mRNA has longer poly(A), in agreement with Table 1; ref. 13]. What we interpret as zone 1 and zone 2 derivatives had mobilities indicative of lengths in the range of 450 to 530 NT and 580 to 700 NT, respectively. Additional, very minor, components in the range of 740 to 830 NT were detectable in the original autoradiogram; they were interpreted as derivatives of zone 3. In total, 11 bands were resolved in this experiment (1a to 1c, 2a to 2e, and 3a to 3c in order of increasing molecular weight).

In a repeat experiment, the same profile of RNase H products was obtained. Individual bands were excised and their RNA was eluted. Upon re-electrophoresis, each component migrated as an individual band (Fig. 4).

Electrophoretic   Analysis   of   RNase   H-Digested [32P]mRNA. Somewhat higher resolution was possible using chorion mRNAs labeled with ^{32}P in culture. Intact *A. pernyi* chorion [32P]mRNA was subjected to electrophoresis, and zones 1 and 2 were recovered separately from the gel. These components were then analyzed by re-electrophoresis, with or without prior digestion with RNase H (Fig. 5).

Prior to RNase H digestion, zones 1 and 2 migrated as very broad but well-defined zones. After digestion, all the components visible in Fig. 3 could be detected. Components 1a to 1c were shown to originate from zone 1, and components 2a to 2e from zone 2. In the original autoradiogram, bands 2a and 2b could each be resolved into two subcomponents; one additional band could be detected at the leading edge of the zone 1 derivatives, and another at the leading edge of the zone 2 derivatives. The pattern shown in Fig. 5 was repeatable in three experiments.

Figure 5 also shows the profile of electrophoretically purified ^{32}P-labeled zones 1 and 2 of *A. polyphemus*, after RNase H digestion. Again, a number of distinct bands can be resolved, but their pattern is distinct from that of the *A. pernyi* components. In particular, the derivatives of *A. polyphemus* zone 1 are more varied in size than the corresponding derivatives of *A. pernyi*.

In the control experiments, the electrophoretic profile of intact chorion mRNAs was not affected by incubation with RNase H in the absence of oligo(dT). Intact and RNase H-treated aliquots of a preparation of ^{125}I-labeled chorion mRNA were analyzed on a 12% polyacrylamide/7 M urea slab gel, in which molecules 10 to 200 NT long can be conveniently resolved. Visual evaluation and scanning of the au-

toradiogram showed that labeled degradation products were not created by RNase H treatment (detection limit in the range 8 to 50 NT, approximately 1–2% of the total RNA). We conclude that the bands seen in Figs. 3 to 5 are generated by removal of the terminal poly(A), rather than by excision of non-poly(A) fragments.

DISCUSSION

In this report we show that digestion of two types of eukaryotic mRNA with RNase H, in the presence of oligo(dT), converts the messages into electrophoretically tighter bands. We interpret this effect as due to removal of poly(A) tails of variable length. Poly(A) is in fact known to be variable in size, apparently shortening as mRNAs age (22).

RNase H-digested preparations are clearly more uniform in size than intact globin mRNA (Fig. 3). The overall decrease in size upon digestion is consistent with recent estimates of the average poly(A) size in this message (23).

In contrast to globin mRNA, chorion mRNA gave rise to a series of discrete bands upon RNase H digestion. This is consistent with the known multiplicity of chorion protein species, and the existence of significant molecular weight differences among them (1). The mobilities on NaDodSO$_4$ gels suggest that the main proteins in each of the two major classes (A and B) differ in length by up to 40 amino acids (1, 2, 21). Correspondingly, in *A. pernyi* the derivatives of zone 1 and zone 2 mRNAs vary in size by approximately 80 and 120 nucleotides, respectively. If the bands seen after RNase H digestion of chorion mRNA in fact correspond to different mRNA species, the parallelism of mRNA and protein sizes (24) might prove to be quite exact, at least for a family of related genes, despite the existence of untranslated regions in the mRNAs (25).

There is no direct proof that an individual mRNA band generated by RNase H corresponds to a distinct mRNA species, coding for characteristic protein component(s). Such proof could be obtained by translation of the bands in a faithful cell-free system, followed by characterization of the

products by peptide maps; it could also be obtained by assaying for nucleotide sequence differences by means of oligonucleotide fingerprints or hybridization reactions.

Despite the absence of such direct proof, there is substantial evidence that the multiplicity of chorion mRNA bands results from the removal of the 3'-terminal poly(A), rather than from coincidental RNA degradation. The evidence is as follows:

(a) We used a homogeneous calf thymus RNase H, that was completely free of ribonuclease activity of the pancreatic type (6, 7). The electropherogram of intact chorion mRNA was unaffected by exposure to RNase H in the absence of oligo(dT). Under our conditions, this enzyme has been shown previously to remove the poly(A) of rabbit globin mRNA, without impairing in the least the template activity of this message (7).

(b) Digestion does not create multiple bands from globin mRNA (Fig. 3).

(c) Upon digestion, the overall decrease in size distribution of chorion ^{125}I-labeled mRNA corresponds to the known average size of the poly(A) (13). Newly synthesized chorion mRNA molecules appear to be digested more extensively (Table 1; also compare the shortening of mRNA in Figs. 3 and 5), but they are also known to have somewhat longer poly(A) (13).

(d) Chorion poly(A) is in fact effectively removed by RNase H digestion, as shown by subsequent probing of the 3' end by polynucleotide phosphorylase (Fig. 2C).

(e) The low molecular weight digestion products contain exclusively adenylic acid (Fig. 1). When only the cytosines of the mRNA are labeled (with ^{125}I), digestion does not create any trichloroacetic acid-soluble radioactive products (Table 1). Thus, low molecular weight oligonucleotides are not generated from any portion of the molecule, other than the poly(A).

(f) Intermediate size message fragments, such as might be excised by RNase H action on internal oligo(A) sequences, are also not produced. The existence of such products was made exceedingly unlikely by the very limited size decrease of the mRNA upon digestion [a decrease predicted by the known size of the poly(A)] and was directly disproved by electrophoretic comparison of intact and digested preparations of ^{125}I-labeled chorion mRNA on a 12% gel.

Our results indicate that the presence of poly(A) causes smearing in mRNA electropherograms, presumably because poly(A) is variable in size. Therefore, effective deadenylylation through the highly specific action of pure RNase H should prove to be a general method for improving the resolution of eukaryotic mRNAs.

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