The Nucleus-limited Hsr-Omega-n Transcript Is a Polyadenylated RNA with a Regulated Intranuclear Turnover

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Abstract. The Drosophila *Hsr-omega* puff, one of the largest heat shock puffs, reveals a very unusual gene, identified by heat shock but constitutively active in nearly all cell types. Surprisingly, *Hsr-omega* yields two transcription end-products with very different roles. The larger, omega-n, is a nuclear RNA with characteristics suggesting a new class of nuclear RNAs. Although it neither leaves the nucleus nor undergoes processing, omega-n RNA is polyadenylated, showing that polyadenylation is not limited to cytoplasmic RNA, but possibly has a function in the nucleus. The amount of omega-n within the nucleus is specifically regulated by both transcription and turnover. Heat shock and several other agents cause rapid increases in omega-n. A rapid return to constitutive levels follows withdrawal of the agents. Degradation of omega-n is inhibited by actinomycin D, suggesting a novel intranuclear mechanism for RNA turnover. Within the nucleus, some omega-n RNA is concentrated at the transcription site; however, most is evenly distributed over the nucleus, showing no evidence of a concentration gradient which might be produced by simple diffusion from the site of transcription.

Previous studies suggested that omega-n has a novel regulatory role in the nucleus. The actinomycin D-sensitive degradation system makes possible rapid changes in the amount of omega-n, allowing the putative regulatory activities to reflect cellular conditions at a given time. Omega-n differs from the best studied nuclear RNAs, snRNAs, in many ways. Omega-n demonstrates the existence of intranuclear mechanisms for RNA turnover and localization that may be used by a new class of nuclear RNAs.

THE heat shock–induced polytene puffs of Drosophila have led to the identification of an important cellular stress response (37). These puffs have also led to the discovery of several families of proteins with unexpected roles as molecular chaperones in both normal and stressed cells. In addition, one of the largest heat shock puffs has identified a very unusual gene, *Hsr-omega* (38). Although discovered because its activity is increased by heat shock, the *Hsr-omega* gene is now known to be constitutively active in nearly every cell type (6). The two major end products of the *Hsr-omega* gene are the RNAs omega-n and omega-c (see Fig. 1). Although these two transcripts share the same initiation site, they are the products of alternate termination and do not have a precursor–product relationship; the nuclear precursor for omega-c is omega-pre-c. Once transcribed, the two *Hsr-omega* products appear to have quite different fates. Omega-c is spliced and exported to the cytoplasm. Omega-n remains unspliced and is retained in the nucleus. Studies of omega-n are revealing unexpected structural and metabolic features of nuclear RNAs that are not precursors to cytoplasmic RNAs but function in the nucleus.

The half-lives of omega-n and omega-c RNA are independently modulated by a variety of agents (4). We have reported that the cytoplasmic RNA, omega-c, turns over rapidly in growing cells but this RNA is stabilized by agents that inhibit protein synthesis at the level of translation. Inhibition of protein synthesis does not have a direct effect on turnover of the nuclear RNA, omega-n. However, levels of omega-n can be increased by several drugs that do not induce the rest of the heat shock response. These drugs have no immediate effect on turnover of omega-c, although they may have secondary effects after longer incubations. When cells are returned to normal conditions, *Hsr-omega* transcripts rapidly revert to normal levels. We have proposed that both omega-n and omega-c have regulatory roles. The rapid changes in RNA levels would make this regulation very responsive to extracellular conditions at any particular time.

Nomenclature: In our earlier reports we named the major *Hsr-omega* transcripts omega-1, omega-2, and omega-3 in order of decreasing size. It has become apparent that this is confusing so the transcripts have been renamed. The new names give an indication of the cellular compartment in which the transcript is found. Omega-n is the large (~10 kb) nucleus-limited RNA (formerly omega-1). Omega-c is the ~1.2-kb cytoplasmic RNA (formerly omega-3). Omega-pre-c is the ~2-kb nuclear precursor of omega-c (formerly omega-2).

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With the exception of the snRNAs involved in RNA processing, little is known about nuclear RNAs that do not serve as precursors for cytoplasmic RNAs. Omega-n is a novel example of an RNA with a role in the nucleus. We believe that there will prove to be other such RNAs with important roles in the nucleus and that omega-n can identify general features of structure and metabolism for this new class of RNA. In this report, we present analyses suggesting that the nucleus-limited omega-n RNA is polyadenylated by the same mechanism used for RNAs destined for the cytoplasm. This raises the possibility that polyadenylation may be used in forming the ends of a large class of nuclear RNAs, just as it is for cytoplasmic RNAs. In addition, we show that levels of omega-n are rapidly modulated in response to extracellular changes. The decrease in omega-n is accomplished by a mechanism that is sensitive to inhibition by actinomycin D, suggesting a novel mechanism of intranuclear turnover. Regulation of RNA turnover in the cytoplasm is becoming recognized as an important component of the regulation of gene expression. Our experiments present the first evidence that regulated turnover can also occur in the nucleus.

Materials and Methods

Treatment of Cells

Cultured diploid *Drosophila melanogaster* (Schneider 3) cells were grown at 25°C in Schneider's Drosophila medium (GIBCO BRL, Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (HyClone Laboratories, Inc., UT). For each experiment, the cells were split into multiple flasks so that each data point in any experiment was from a flask derived from the same parent culture. Since the response of the cells to heat shock and recovery is influenced by nutritional state, cell density, etc., in all cases quantitative comparisons have only been made between cells from the same experiment. Qualitative conclusions were entirely reproducible. For the prolonged heat shock experiments, individual flasks were incubated for the desired period of time at 36°C in the presence or absence of 1 μg/ml actinomycin D (Sigma Chemical Co., St. Louis, MO) prior to RNA extraction. Recovery from heat shock was examined by exposing cells to a brief (30 min) heat shock at 36°C after which they were allowed to recover for 0–4 h at 25°C in the presence or absence of the drug. Actinomycin D was added at a concentration of 1 μg/ml immediately upon return to 25°C or for the last half of the recovery period at 25°C. The effect of prolonged exposure to actinomycin D was also examined. In this case, cells were maintained at 25°C for 0–4 h in the presence or absence of the drug.

RNA Extraction

Total cellular RNA was extracted from Schneider 3 cells using the guanidine-HCl procedure originally described by Cox (11) with some modifications. Briefly, 2.5–5 × 10^8 cells were pelleted and resuspended in ~0.2 ml of medium. The suspension was slowly added to 2 ml of 7.5 M GuHCl, 0.025 M NaAc, 0.1 M Tris base, pH 7.0, and 0.01 M DTT with vortexing. One half volume of ethanol was added to this suspension which was then incubated at −20°C overnight. Nucleic acids were pelleted by centrifugation at 5,000 *g* for 10 min at 4°C. The pellet was resuspended in 2 ml of 8 M GuHCl, 0.025 M NaAc and precipitated with one half volume ethanol at −20°C for 3–4 h. Nucleic acids were pelleted by centrifugation as above and washed twice with 70% ethanol. The final pellet was resuspended in 0.1% SDS, phenol/chloroform extracted, and precipitated with 2 vol of 4.5 M NaAc pH 6.0 at 4°C overnight. RNA was pelleted by centrifugation at 12,000 *g* for 15 min at 4°C. The pellet was washed with 70% ethanol and dissolved in a small volume of sterile H_2O.

Northern Blot Analysis

RNA was denatured by treatment with glyoxal as described in Sambrook et al. (40), separated on 1% agarose gels and blotted to nylon membrane according to Thomas (44). Filters were probed with ^{32}P-labeled (17) gel-purified DNA fragments as described by Garbe and Pardue (19).

Filters were exposed to X-ray film as well as imaged and the hybridization quantitated using a Betascope (Betagen Corp., Framingham, MA). The amount of ^{32}P incorporated into specific RNAs was normalized against the level incorporated into the appropriate control RNA (25°C). These normalized values were then plotted as a function of treatment. All filters have been quantified to test conclusions drawn by visual inspection. We have chosen to present photographic prints of autoradiograms because they allow easier visual integration of information. In all cases, the Betascope data confirm the photographic results (see sample Betascope data in Figs. 4, 5, and 7).

In Situ Analysis of RNA

Salivary glands were dissected in DPBS (10 mM sodium phosphate, pH 7.4, 130 mM NaCl) plus 0.05% NP-40 (total 1 min), and fixed for 1 min in 3.7% formaldehyde in DPBS. Glands were softened 5 min in 45% acetic acid and squashed. Preparations were frozen in liquid nitrogen and coverslips were flipped off. Slides were then plunged into 95% ethanol and air dried. Cultured cells were cytocentrifuged onto slides subbed with 25% subbing solution (35), fixed 30 min in 3.7% formaldehyde in DPBS, washed 2 × 5 min in DPBS, permeabilized for 5 min in 0.2% Triton X-100 in DPBS, and washed in DPBS. Both salivary gland and cultured cell preparations were acetylated for 10 min in 5 ml/liter acetic anhydride in 0.1 M triethanolamine, pH 8.0, washed in DPBS, dehydrated through ethanols, and dried. ^{3}H-labeled probe was added in 50% formamide/2× SSPE (1× SSPE: 150 mM NaCl, 10 mM NaH_2PO_4, pH 7.4, 1 mM EDTA, pH 7.4) and allowed to hybridize overnight at 50°C. Probe was removed by washing in 2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature and slides were treated with 20 μg/ml RNase A at 37°C for 30 min. Slides were washed in 2× SSC, dehydrated, dried, and autoradiographed.

The probe for omega-n was a T7 polymerase transcript of the non-transcribed strand of the 284-bp *Hsr-omega* repeats. Both in situ hybridization and DNA blots show this sequence to be specific for the *Hsr-omega* locus (data not shown). The probe for the ribosomal RNA precursor was a T7 polymerase transcript of the non-transcribed strand of the external transcribed region from the nuclear precursor of *D. melanogaster* ribosomal RNA (14).

Isolation and Sequence Analysis of the 3′ End of Hsr-omega

A 1,460-bp SnaBI-HindIII fragment from a clone encompassing the entire *Hsr-omega* gene was subcloned into pGEM3Z to produce clone pDm152. This fragment contains the last repeats of omega-n plus approximately 775 nucleotides 3′ to the end of the last repeat. This fragment was identified as the most 3′ fragment by RNA blot hybridization. Subclones derived from this fragment confirmed this identification. Both the subcloned restriction fragments and fragments generated by deletions of the 1,460-bp SnaBI-HindIII fragment were used for sequence analyses. Both strands of the 1,460-bp fragment have been sequenced.

RNase Protection Assay

From 1.0–50.0 μg of total cellular RNA was used in each assay (41) initially to determine the amount of RNA necessary for binding. Total cellular RNA was precipitated and resuspended in 30 μl hybridization buffer (40 mM Pipes, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl, 80% formamide). Approximately 1 × 10^5 cpm of gel-purified anti-sense RNA generated by Sp6 RNA polymerase from a subclone encompassing the extreme 3′ end of omega-n (see Fig. 3) inserted into pGEM3Z was added to tubes containing varying amounts of total cellular RNA. The mixture was incubated at 85°C for 10 min to denature the RNAs, transferred to a 45°C H_2O bath and incubated for 8–12 h. Following incubation, the mixture was first treated with RNases A and T1 for 1 h at 30°C, followed by treatment with proteinase K. The samples were phenol/chloroform extracted, precipitated with ethanol, resuspended in a small volume of formamide loading buffer (80% formamide, 10 mM EDTA, pH 8.0, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue), heated to 95°C for 5 min, and loaded onto a 7 M urea/4% polyacrylamide gel. Electrophoresis was for approximately 4 h at 35 W. Following electrophoresis, the gels were fixed for 20 min in 10% MeOH, 10% acetic acid, after which they were dried at 80°C, and apposed to X-ray film.

3' End Sequence

The Accession No. for the cloned 3' end of omega-n (clone pDm152) is U02277.

Results

The Omega-n Transcript Is Broadly Distributed Over the Nucleus in Both Heat Shock and Control Cells

Both in situ hybridization (Fig. 2) and cell fractionation studies (20) show that omega-n is limited to the nucleus. Athough the sequences of the 5'-most 2 kb of this RNA are shared with the cytoplasmic omega-c and its precursor, omega-pre-c, the 3' tandem repeats are found only in omega-n. Since these repeats, which are specific to the *Hsr-omega* locus, make up >75% of the length of omega-n, they provide an excellent probe for cytological localization. In situ hybridization to RNA within many kinds of cells shows that, with the exception of a concentration over the locus of transcription, the omega-n RNA tends to be evenly distributed over the nucleus. The overall pattern of localization is not changed by heat shock or other agents; however, the total amount of the transcript is increased by these agents.

When cultured Drosophila cells were centrifuged onto slides, omega-n RNA was detected as small clusters of silver grains over the nuclei after short autoradiographic exposures for a ^{3}H-labeled probe. Studies of polytene nuclei (see below) indicated that these clusters marked hybridization to RNA over the locus of transcription in polytene region 93D. After longer autoradiographic exposures, omega-n was detected fairly evenly over the rest of the nucleus, obscuring the clusters. In the centrifuged cultured cells this distribution was seen as a ring of hybridized probe around the large central nucleolus (Fig. 2 *B*). The ring included most of the nucleoplasm of these tiny cells. Although the nucleolus did not contain omega-n RNA, it could be seen to contain precursors to ribosomal RNA when probed with RNA complementary to the external transcribed region of the rRNA precursor (Fig. 2 *C*). In situ hybridization with probes that detect hsp83 mRNA showed that it was distributed over the cytoplasm of these cells (Fig. 2 *A*). No nuclear clusters were observed.

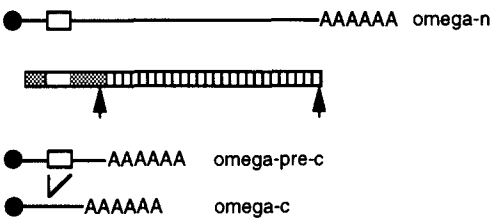

Figure 1. Schematic diagram showing the structure and transcripts of *Hsr-omega*. The cross hatched boxes represent exons 1 and 2 while the large open box defines the sequences that are spliced out to make omega-c. Together these boxes comprise the unique region. The series of small open boxes represent the >8-kb segment of tandem direct repeats. The arrows mark the polyadenylation sites. All three transcripts have the same transcriptional start site as is indicated by the closed circles. The largest transcript, omega-n, is colinear with the entire transcription unit containing approximately 3 kb of unique region and >8 kb of tandem repeats. Omega-n is limited to the nucleus. The second transcript, omega-pre-c, which is made by alternative termination near the first polyadenylation site, is also nuclear. However, this transcript does not accumulate in the nucleus but is rapidly spliced and transported to the cytoplasm to yield the third transcript, omega-c. Two different probes have been used in this study. The omega-c sequence detects all *Hsr-omega* RNAs while the 3' repeat sequence (two repeats from within the >8 kb of tandem repeats) detects only omega-n.

Polytene nuclei from salivary glands present an opportunity to examine much larger interphase nuclei. Results from in situ hybridization to salivary gland cells were entirely consistent with results from diploid cultured cells. Short autoradiographic exposures showed a single cluster of silver grains over each nucleus. When chromosomes were spread enough to allow analysis of the banding pattern, it was clear that this cluster was over the 93D locus from which omega-n is transcribed. Because homologous chromosomes are paired in these cells, there was only one cluster per nucleus (Fig. 2 *D*).

Longer autoradiographic exposures revealed that, in addition to the concentration of omega-n over 93D, there was a lower level of the RNA spread over the rest of the nucleus (Fig. 2 *D*). The pattern of hybridized RNA did not show the concentration gradient that might be expected if the RNA merely diffused from the site of transcription through the dense intranuclear material of this large cell. Except at the site of transcription, the localization of omega-n did not seem to be due to association with chromatin. In broken nuclei (Fig. 2 *E*), the hybrid remained in the area of the nuclear contents but was not attached to the chromosomes.

Polyadenylation of Omega-n RNA Utilizes RNA Processing Signals Typical of Pre-messenger RNA

The early evidence indicating that omega-n was polyadenylated was surprising because omega-n is a nuclear RNA (29). Previously studied poly(A)$^+$ RNAs had all been either cytoplasmic messenger RNAs or precursors to such RNAs (45). Although omega-n remains in the nuclear compartment, sequence analysis of the transcription unit suggests that this RNA is polyadenylated by the same mechanisms that polyadenylate mRNA precursors.

Multiple polyadenylation signals (AAUAAA) were detected by sequence analysis of the 3' end of the *Hsr-omega* transcription unit (Fig. 3 *A*). RNase protection analyses showed that omega-n RNA uses at least three termination signals which produce RNAs with different 3' ends. These RNAs yielded protected fragments of 530, 365, and 160–170 bp when antisense probe was hybridized to total cellular RNA and the hybrids were digested with RNases A and T1 (Fig. 3 *B*). The transcripts defined by the protected fragments of 530 and 160–170 bp appear to utilize classic 3' end processing signals. In both cases, the hexanucleotide sequence (AAUAAA) is found at an appropriate distance upstream of the poly(A) addition site (i.e., cleavage site) while a less conserved GU-rich sequence is located downstream. These studies suggest that the polyadenylation signal at position 865 (defined by the 160–170-bp protection fragment) is used more frequently than that at position 1286 (defined by the 530-bp protection fragment). The RNA defined by the 365-bp protection product suggested use of an atypical polyadenylation site, possibly the CAUAUA sequence at position 1072. Several Drosophila mRNAs are defined by multiple 3'

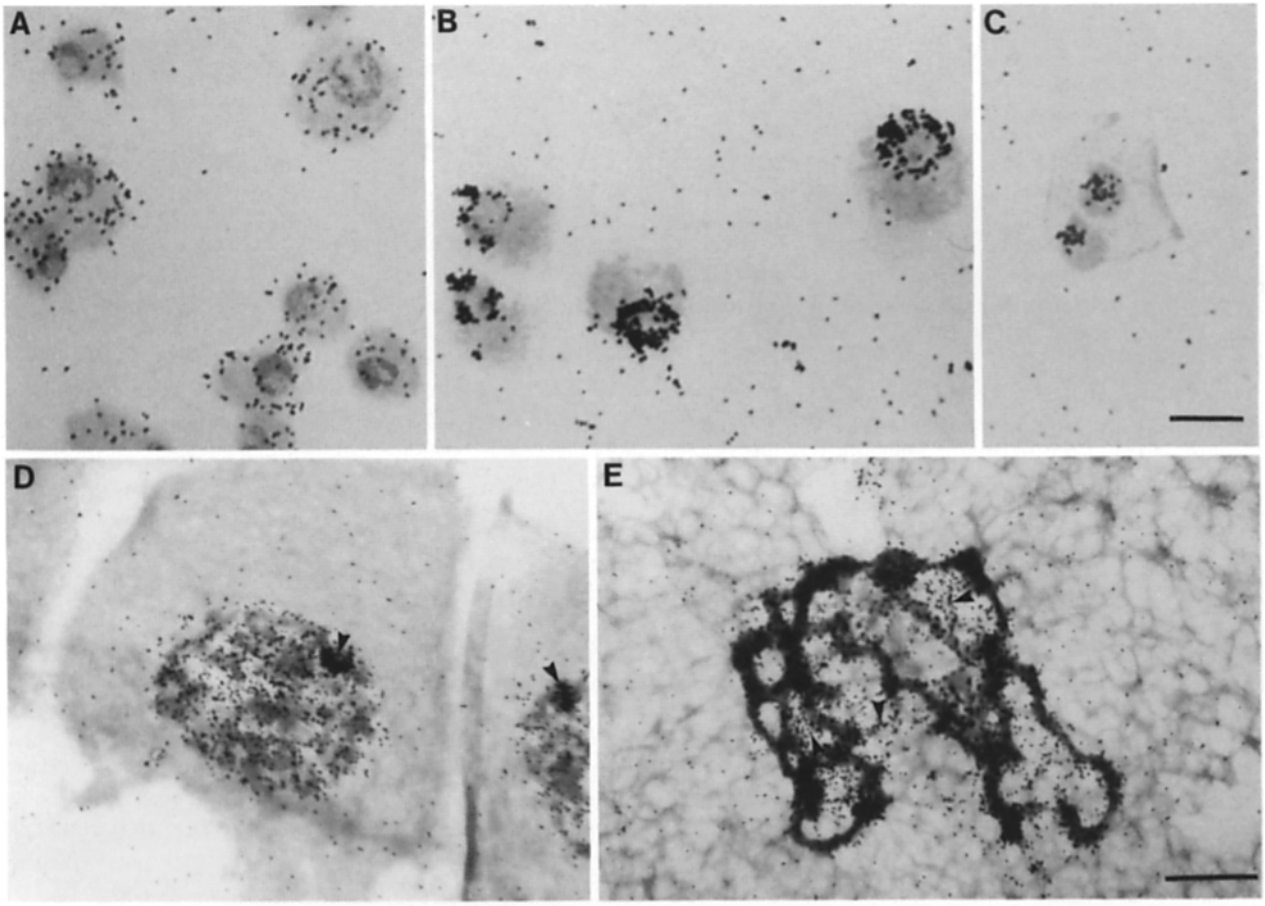

Figure 2. Autoradiograms showing intracellular localization of specific RNAs in diploid and in polytene cells. (*A*) Cultured (Schneider 3) cells probed with ^{3}H-labeled RNA complementary to hsp83 RNA. The mRNA is detected throughout the cytoplasm. Nuclear areas are nearly free of silver grains because little hsp83 RNA is in nuclei and the cytoplasmic layer over the nuclei is very thin. (*B*) Schneider 3 cells probed with ^{3}H-labeled RNA complementary to 3′ repeats of omega-n. The omega-n RNA is detected only in the nuclei and appears rather evenly spread around the large central nucleolus. These cells have been heat shocked at 36°C for 1 h. This treatment has increased the amount of omega-n RNA but has not affected the distribution of this RNA within the nucleus. (*C*) Binucleate cell probed with ^{3}H-labeled RNA complementary to the external transcribed region of the precursor to ribosomal RNA. In each nucleus, rRNA precursor is detected within the nucleolus. (*D*) Polytene salivary gland cells probed with ^{3}H-labeled RNA complementary to the 3′ repeats of omega-n. As in diploid cells (*B*), the omega-n RNA appears to be limited to the nucleus. With the autoradiographic exposure chosen for this photograph, the concentration of omega-n RNA over its site of transcription (*arrowheads*) is easily seen in both this nucleus and the partial nucleus of the adjacent cell. The rest of the transcript is spread evenly over the nucleus, showing no evidence of decreasing concentration with distance from the site of transcription. (*E*) This polytene nucleus was broken open when the preparation was made. The silver grains (*arrowheads*) can be seen clustered between the darkly stained chromosomes. The omega-n RNA detected by the probe has remained in the nuclear area but does not appear to be specifically associated with the chromosomes, suggesting that the nuclear distribution is determined by some other component of the nucleus. This nucleus is from a heat-shocked larva but the distribution of omega-n is similar in control cells. Bars: (*A–C*) 1 μm; and (*D* and *E*) 2 μm.

ends and in some cases one or more ends can be directed by an atypical polyadenylation signal. For example, a CAUAUA sequence is also found at an appropriate distance upstream of one of the defined cleavage sites for mp20, a muscle specific Drosophila gene exhibiting alternative termination during development (2). Omega-n is expressed in almost all Drosophila tissues and stages (6). It is not known whether it undergoes tissue-specific or developmentally regulated alternative polyadenylation. Although the CAUAUA seems the most attractive possibility, an AAUAAU sequence at position 974 or an AGUAAA sequence at position 1031 could also be used since these sequences have either been identified as putative polyadenylation signals in nature (25) or shown to act as polyadenylation signals in in vitro studies (42). The two sequences are somewhat further upstream from the cleavage site than is typical for polyadenylation signals, however the distance between the AAUAAA sequence and the polyadenylation site can be variable (45).

Levels of Omega-n Are Regulated by Both Transcription and Turnover

The *Hsr-omega* locus at 93D was originally identified because heat shock and several other agents induced a puff in polytene chromosomes, suggesting that transcription was turned on by the treatments (37). When the locus was cloned and used as a probe to measure transcript levels, it became apparent that even cells with unpuffed chromosomes con-

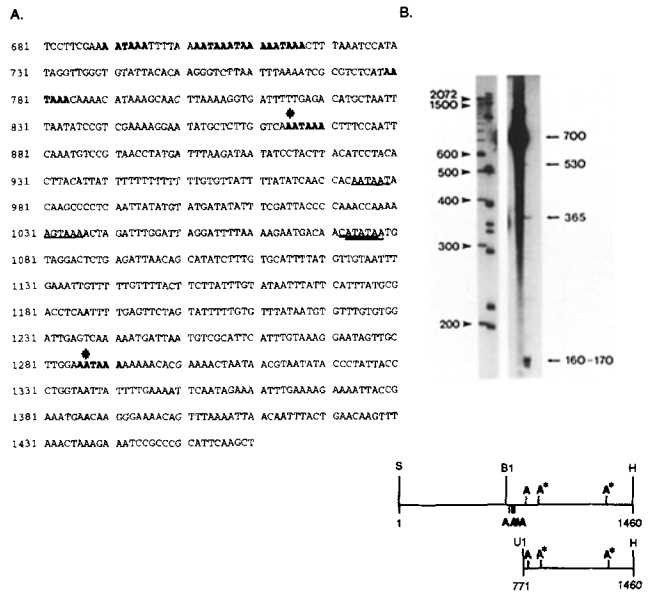

Figure 3. Sequence analyses and RNase protection analyses suggest that the nucleus-limited transcript, omega-n, is polyadenylated by typical polyadenylation signals. (*A*) Sequence analysis of the 3′ end of omega-n RNA. Classic polyadenylation signals are in bold. Those signals at position 865 and position 1286 (each marked by an asterisk) define the 160–170- and 530-bp protection fragments observed in the RNase protection analysis respectively (*B*). Putative alternate polyadenylation signals are underlined. This sequence corresponds to the region between BstBI (*BI*) and HindIII (*H*) in the schematic in *B*. (*B*) RNase protection analysis shows that omega-n RNA is defined by at least three termination signals. The schematic below the autoradiogram shows both the 1,460-bp SnaBI (*S*)-H fragment derived from the original 1.5-kb SnaBI–EcoRI fragment and a subcloned BstUI (*UI*)-H fragment that was used to generate antisense RNA for the RNase protection analysis shown above. The A's in bold mark the positions of the classic polyadenylation signals (bold print in *A*). Those A's with an asterisk define the polyadenylation signals at positions 865 and 1286. The first two lanes of the autoradiogram contain molecular weight markers. The third lane contains hybrids (which have not been digested with RNase A or T1) between total cellular RNA and ^{32}P-labeled RNA generated from the UI-H subclone. A predicted RNA size of 689 bp is in good agreement with the observed size of 700 bp. The final lane contains samples after digestion with RNases A and T1. The protected fragments of 160–170 bp and 530 bp suggest that cleavage occurs just downstream from the polyadenylation signals at positions 865 and 1286, respectively (marked by the asterisks in the schematic and in A). The 365 bp protection fragment suggests use of an atypical polyadenylation signal. Several potential candidates are underlined (*A*). These signals occur at appropriate distances upstream of the cleavage site that is defined by the 365-bp protection fragment. Anti-sense RNA generated from a series of 3′ deletion constructs all ending at the B1 site in the schematic confirmed these findings (data not shown).

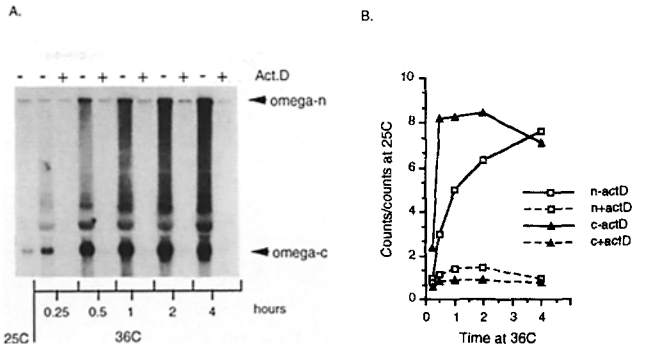

Figure 4. Autoradiogram showing accumulation of RNA during continuous heat shock in the presence (+ sign above each lane) or absence (− sign above each lane) of actinomycin D. The drug inhibits the heat shock-induced rise in amount of both omega-n and omega-c; however, it also inhibits turnover of omega-n but not omega-c. Schneider 3 cells were held at 25°C or heat-shocked at 36°C for the time (*hours*) indicated prior to RNA extraction. (*A*) Total cellular RNA was gel-fractionated, blotted, and probed with ^{32}P-labeled omega-c sequence. The omega-c sequence is common to all the *Hsr-omega* RNAs (Fig. 1) and therefore can be used to detect the *Hsr-omega* RNAs with equal efficiency. (*B*) Graphical presentation of data following Betascope analysis of the filter that gave rise to the autoradiogram in *A*. The amount of ^{32}P hybridized to omega-n and omega-c RNA from treated cells was normalized against the level hybridized to RNA from control cells (data not shown). These normalized values were plotted as a function of time at 36°C.

tained some *Hsr-omega* RNA. Evidently the puffing is an indication that transcription has been greatly increased over the constitutive levels, rather than an indication that transcription is turned on.

Approximately 400 bp 5′ to the start of transcription of the *Hsr-omega* locus has been sequenced for *D. melanogaster*, *Drosophila hydei*, and *Drosophila pseudoobscura (21)*. Each of these regions has several sequences which can be fit to the consensus heat shock element, suggesting that the locus is regulated, at least in part, by the heat shock transcription factor (7). Heat shock does in fact cause puffing of polytene locus 93D and also results in a large increase in the level of omega-n RNA detected in cell extracts. That this increase is due to an increase in transcription of the RNAs was suggested by treatment of cells with actinomycin D (Fig. 4). During prolonged heat shock, the levels of both omega-n and omega-c continued to increase. Within 15 min of treatment with actinomycin D, omega-c was no longer detected, indicating that transcription was inhibited in the presence of the drug. Since all omega transcripts come from the same transcription start site, omega-n transcription should also be stopped by the drug. When cells were exposed simultaneously to heat shock and actinomycin D, the levels of omega-n remained comparable to those detected in control cells (Fig. 4, *A* and *B*). Therefore, actinomycin D prevented the increase in omega-n RNA observed in cells exposed to heat shock without the drug. Since transcription is blocked by actinomycin D, the omega-n RNA detected in cells treated with the drug must represent pre-existing transcript. Omega-n RNA is therefore much more stable than omega-c RNA which is generated from the same transcription start site. Experiments described below suggest that this stability is due, at least in part, to inhibition of turnover by actinomycin D. Omega-n RNA continued to accumulate over 4 h of heat shock at 36°C. Treatment with actinomycin D blocked this accumulation as expected if actinomycin D is inhibiting transcription of omega-n.

As a control for the heat shock conditions and drug treatment used in these experiments, we probed parallel filters with sequences from two other heat shock genes, hsp83 and

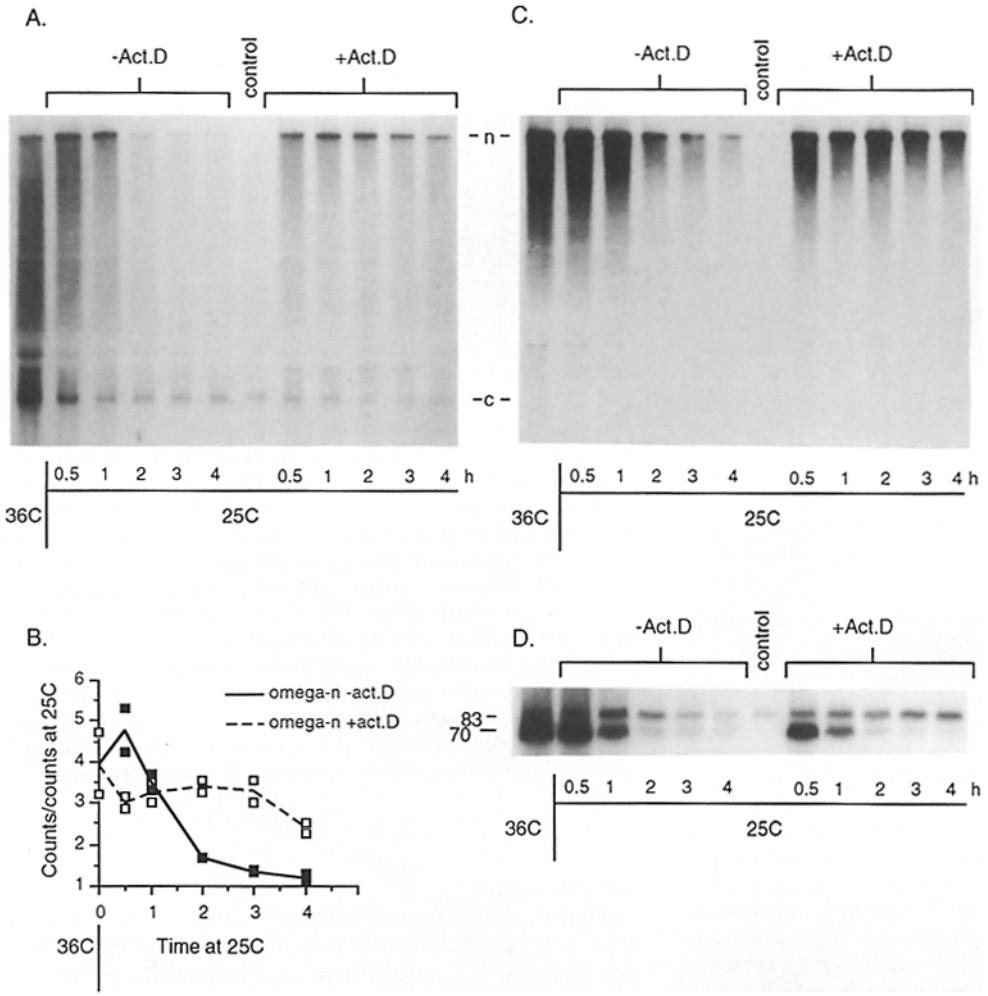

Figure 5. Autoradiogram showing the changes in RNA levels as cells recover from heat shock. The turnover of omega-n RNA, but not omega-c RNA, is significantly inhibited by addition of actinomycin D during recovery. Schneider 3 cells were heat-shocked at 36°C for 30 min and then returned to 25°C, at which time actinomycin D was added to half the cultures. Total cellular RNA was extracted from cells after the indicated recovery times at 25°C (*h*). (*A*) RNA blots probed with omega-c sequence. Both omega-n (indicated by the letter *n*) and omega-c (indicated by the letter *c*) RNA levels rapidly return to control values during recovery in the absence of actinomycin D. This data is represented graphically in part B. (*B*) Plot of Betascope analysis of the filter that gave rise to the autoradiogram in *A* and a second filter from a duplicate experiment. Normalized data are plotted as a function of time at 25°C. (*C*) RNA blot of the same samples analyzed in *A* except that this blot was probed with the omega-n repeat sequence which does not detect omega-c. The conclusions about omega-n stability do not change when the measurements are made with the repeat sequence; however the smear of partially degraded molecules below the full length omega-n is more evenly distributed over the entire size range when the blot is probed with omega-c sequence while more hybridization to partially degraded molecules is seen toward the top of the lane when the blot is probed with the repeat sequence. This difference in detection of degraded molecules would be expected if repeats are preferentially lost before the 5′ end and argues that omega-n degradation begins at the 3′ end. (*D*) Segment of duplicate filter probed with sequences complementary to hsp70 (*70*) and hsp83 (*83*) mRNA. Hsp70 mRNA is degraded rapidly when cells are returned to 25°C whether actinomycin D is present or not. Hsp83 mRNA returns to control values more slowly and its turnover appears to be inhibited by actinomycin D.

hsp70. These two mRNAs continued to accumulate with heat shock. Treatment with actinomycin D prevented this accumulation, showing that the conditions used for these experiments were adequate (data not shown).

The Turnover of Omega-n Is Regulated by a Mechanism That Is Sensitive to Actinomycin D

One of the distinctive features of omega-n is the rapidity with which it can be returned to control levels when the inducing treatment is stopped (Fig. 5). This turnover appears to be under the control of a novel mechanism that is sensitive to actinomycin D. In all of our experiments, the addition of actinomycin D caused the omega-n RNA to be stabilized at approximately the level it had reached when the drug was added. This stabilization happened whether the cells were in the process of increasing (Fig. 4), decreasing (Fig. 5), or simply maintaining the levels of omega-n (Fig. 6).

The experiments described in the preceding section have shown clearly that actinomycin D inhibits the transcription of both omega-n and omega-c. Those experiments also gave the first hint that the drug had a differential effect on the turnover of these two transcripts; omega-c turned over rapidly after addition of the drug while omega-n remained more or less unchanged in cells treated with actinomycin D. Earlier experiments had shown that omega-c RNA was affected by inhibitors of protein synthesis. None of the protein synthesis inhibitors used in those experiments affected the turnover of omega-n (4).

In cells subjected to a brief heat shock at 36°C and then allowed to recover at 25°C, omega-n RNA levels decreased rapidly to control levels. In parallel cultures treated with actinomycin D during the recovery period, it was apparent that the level of omega-n RNA remained virtually unchanged over this 4-h period. A slight decrease was observed after 4 h (Fig. 5 *B*). However, this decrease could reflect degradation due to stress and cell death ensuing from prolonged incubation of the cells with the drug. Treatment with actinomycin D resulted in the rapid disappearance of omega-c,

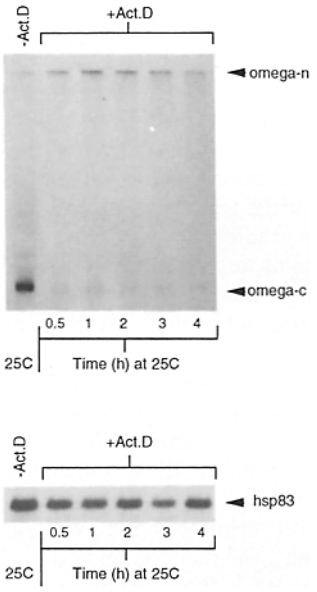

Figure 6. Autoradiogram showing that omega-n RNA, but not omega-c RNA, is stabilized after actinomycin D treatment at 25°C. Schneider 3 cells growing at 25°C were treated with actinomycin D (1 μg/ml) for the times indicated below each lane. RNA was then extracted, gel-fractionated, blotted, and the filter probed with omega-c sequence. A portion of the filter probed for the constitutive hsp83 mRNA is shown below. Almost all of the omega-c RNA disappears rapidly after addition of the drug but the levels of omega-n remain nearly unchanged, as do levels of hsp83 mRNA.

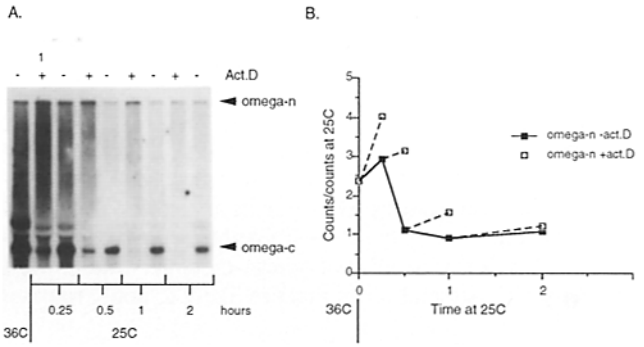

Figure 7. Actinomycin D can inhibit the turnover of omega-n at any time during the recovery from heat shock. Schneider 3 cells were heat-shocked and allowed to recover at 25°C as in Fig. 6. Actinomycin D was added (+) to some cultures but not others (−). In each case the drug was added for the last half of the recovery period as is indicated by dotted lines in the graph in B. The exception is lane *1* (*+1*) where drug was added immediately on return to 25°C. (*B*) Plot of Betascope analysis of the filter in (*A*). Normalized values were plotted as a function of time. Solid lines connect points from cultures with no actinomycin D. Dotted lines connect times when actinomycin D was added to a culture with the time at which RNA was prepared from that culture. In each case, the amount of omega-n in cultures treated with actinomycin D appears to have changed little after addition of the drug while untreated cultures continue to turn over this RNA. Compare each actinomycin D–treated culture with the untreated culture that was assayed just when actinomycin D was added to that particular culture. The actinomycin D rapidly changes the slope of the decay curve.

consistent with the earlier observation that actinomycin D had no effect on the turnover of this RNA. The stability of histone mRNAs was also unaffected by the drug (data not shown).

We have probed our RNA blots with the omega-c sequence. As is shown in Fig. 1, this sequence is common to each of the three transcripts, occurring once per molecule. Therefore, this probe allowed us to measure all three *Hsr-omega* RNAs with equal efficiency. When filters were probed with the repeat region which is specific for the 3′ end of omega-n, we obtained the same results for the turnover of intact omega-n RNA (compare Fig. 5, *A* and *C*). There was, however, a distinct difference in the patterns of the partially degraded RNAs when the two different probes were used. Omega-c sequences hybridized rather evenly to the RNAs between 10 and 2 kb while the repeat hybridized to a declining gradient. Such a pattern would be expected if the repeat region were preferentially removed first, suggesting that degradation of the omega-n RNA is initiated at the 3′ end.

The effect of actinomycin D on the recovery of hsp83 and hsp70 mRNAs was also examined (Fig. 5 *D*). Hsp70 mRNA declined rapidly when cells were returned to 25°C, consistent with the rapid repression of hsp70 translation and concomitant degradation of hsp70 mRNA as reported by DiDomenico et al. (15, 16). This decrease in hsp70 mRNA did not seem to be affected by actinomycin D. In contrast, hsp83 mRNA levels, like omega-n, were stabilized in the presence of actinomycin D. However, in the case of hsp83, the drug is inhibiting the turnover of a cytoplasmic mRNA. Like *Hsr-omega*, the hsp83 gene is transcribed constitutively and transcription is elevated in heat shock. The mRNA levels rise more slowly than those of omega-n during heat shock and fall more slowly during recovery (4). Whether any relationship exists between the stabilization of the omega-n nuclear RNA and the hsp83 mRNA is unclear.

Further evidence for regulation of omega-n RNA at the level of turnover was provided when RNA was extracted from nonstressed cells treated with actinomycin D (Fig. 6: see also reference 4). Under these conditions, omega-c RNA dropped dramatically within the first 15 min. A very low level of omega-c RNA persisted throughout the treatment and may represent a subset of cells resistant to actinomycin D. In contrast, omega-n RNA increased slightly within the first 15 min of treatment and was maintained at this level for the remaining 3 h. The initial increase may reflect transcription prior to blockage by the drug. Hsp83 mRNA persisted throughout the experiment consistent with the behaviour of this RNA during recovery from heat shock (Fig. 5 *D*).

The decreased turnover of omega-n RNA in the presence of actinomycin D was independent of the point in recovery when the drug was applied to the cells (Fig. 7). Addition of actinomycin D caused the omega-n RNA to be stabilized at approximately the level it had reached when the drug was added to the cells, suggesting that some aspect of the turnover mechanism is very unstable and requires ongoing transcription. As in earlier experiments, the amount of omega-n RNA increased initially. Once again, we believe this increase reflects the time it takes for actinomycin D to block transcription.

Because these experiments were done by adding actinomycin D to intact cells, they do not identify the exact mechanism of action by which actinomycin D stabilized omega-n RNA. However, analyses of the effect on other RNAs showed that, whatever the mechanism, it did not affect all types of RNA. Furthermore, the mechanism is one that is extremely responsive to the addition of the drug since stabilization happened under a variety of cellular conditions. During heat shock the levels of omega-n vary greatly, depending on the temperature of the heat shock and its duration. Turnover of this RNA during recovery could serve as an important

regulatory mechanism. Modulation of this sort is key to the regulation of a variety of genes (1).

Discussion

Except for snRNAs, omega-n is the first nucleus-limited RNA to be characterized. At present, omega-n exhibits several novel characteristics. However its apparent novelty may only reflect the fact that nuclear RNAs have received very little attention. These studies of omega-n demonstrate aspects of nuclear RNA metabolism that are likely to apply to other RNAs.

Although the *Hsr-omega* locus has many unusual properties, each of the many Drosophila species studied has one member of the set of heat shock puffs that has conserved these unusual properties (36). This conservation argues that the locus has a function. The function must be a novel one; one that has come to our attention because of the heat shock puff, but one that is also important in non-stressed cells. Our earlier studies have suggested that two of the major transcripts of *Hsr-omega*, omega-n and omega-c, are, in fact, the end products of the locus and that these are regulatory RNAs (4, 18). Omega-n appears to act in the nucleus and omega-c appears to act in the cytoplasm. The two RNAs are transcribed from the same transcription start site but have different termination sites. If the alternative termination is unbiased, the shared start could be a mechanism for ensuring equal starting amounts of the two RNAs.

The study reported here concentrates on the structure and post-transcriptional regulation of omega-n. Sequence analyses of this RNA from different Drosophila species suggest that a major feature of omega-n structure is the >8 kb of tandem repeats that make up the 3′ end of the RNA in each Drosophila species. These repeats are not shared with the other transcripts of this locus. The repeats vary in size (e.g., 284 bp in *D. melanogaster*, 115 bp in *D. hydei*) and sequence. Although the repeats are very conserved within each species (less than 10% divergence; 5), the only feature conserved across species is a nine nucleotide sequence, AUAGGUAGG, which occurs at approximately 100-bp intervals in each RNA, regardless of the repeat size. The nonamer could be a protein binding site. Omega-n RNA may act by binding a protein, thereby modulating its activity or forming a structure. We have identified a candidate protein which we are now in the process of isolating. Such binding could serve as a very efficient way of regulating an abundant protein since the levels of omega 1 are rapidly elevated and decreased in response to a number of agents (5). We note that, if omega-n forms a structure with the protein, omega-n must act in a regulatory role in the sense that the amount of structure will vary as the amounts of omega-n change.

The localization of omega-n differs in two ways from that of other transcripts studied in similar experiments (mRNAs encoding alpha tubulin, histone, hsp83, and hsp70). First there is a significant concentration of omega-n RNA over the site of transcription in both diploid and polytene cells. This concentration may simply reflect the large size of the RNA (>10 kb) which requires longer to transcribe than the other RNAs studied and therefore could result in a large target for probe at 93D. However, the concentration may also indicate slower processing or assembly of an RNP; that too, could increase the mass of omega-n concentrated at 93D if all processing occurs at that site. Large (100–300 nm) RNP complexes have been shown to accumulate in the *Hsr-omega* puff following heat shock (12), suggesting that there is complex assembly at this locus.

The second unique feature of omega-n localization is the even distribution over the rest of the nucleus. This distribution does not seem to be part of a concentration gradient extending away from the cluster at the transcription site. The failure to detect a concentration gradient suggests that, after release from 93 D, omega-n is transported rapidly compared to its turnover. We do not detect the numerous discrete "transcript domains" that have been seen when nuclei from many organisms are probed for poly(A)$^+$ RNA (10). Since poly(A) localizations have not been analyzed in Drosophila, it is not clear whether the more uniform distribution seen for omega-n is specific for omega-n, an unusual RNA, or whether it is a general feature of poly(A)$^+$ RNA in the Drosophila cells that we have studied.

The localization of omega-n transcripts within the nucleus is qualitatively the same in both heat-shocked and control cells although the amounts of the RNA differ significantly in the two cases (our unpublished observations). We interpret these observations to imply that omega-n performs similar functions in the two conditions but that more activity is needed during heat shock.

Although transcription of *Hsr-omega* is increased along with the other heat shock genes, transcription at *Hsr-omega* is also controlled by factors that do not affect other heat shock genes. Drugs such as colchicine, colcemid, and benzamide specifically induce *Hsr-omega* puffing (27, 28); however the puff is somewhat inhibited when exposed to the inducing agents in combination or in sequence, suggesting that the locus is autoregulated. In addition to the complex transcriptional regulation, *Hsr-omega* shows complex regulation of RNA turnover. This dual regulation makes the levels of *Hsr-omega* transcripts very responsive to the immediate changes in cellular conditions, an attractive trait for RNAs that act as regulatory molecules.

Our studies of omega-n show that there are mechanisms within the nucleus that can regulate the turnover of nuclear RNAs. The turnover of omega-1 can be quite fast if cells are returned immediately to control conditions. In addition, the turnover seems to be under continuous control. If actinomycin D is added at any time during the recovery, omega-n levels approximate those observed at the point of drug addition. Although these experiments have not defined the mechanism by which actinomycin D is acting, it seems unlikely that it is preventing transcription of a short-lived RNA that encodes a short-lived protein, as has been suggested for the inhibition of creatine phosphokinase (39), human transferrin receptor (31), and *c-fos* (43). In these cases, turnover is rapidly inhibited by cycloheximide while inhibition of protein synthesis does not affect omega-n (4). The continuous control of degradation allows a sensitive modulation of omega-n RNA levels that contrasts with the robust induction of heat shock protein synthesis. Lewis et al. (30) found that the addition of actinomycin D 5 min after the start of a 37°C heat shock had little, if any, effect on the amount of heat shock proteins present at 45 min. The RNA induced in the early stages of heat shock is apparently stable enough to direct substantial protein synthesis even after actinomycin D has blocked further transcription. In contrast, some aspect of the mecha-

nism which leads to a decrease in omega-n on recovery is not stable and therefore the recovery does not proceed after the addition of actinomycin D.

The early studies on the 93-D heat shock locus showed that the nuclear RNA bound to oligo-(dT) and thus appeared to be polyadenylated (29). Finding a nuclear RNA that was polyadenylated was unexpected. At that time all of the known poly(A)$^+$ RNAs were mRNAs or the nuclear precursors of mRNAs. With the notable exception of the mRNAs for the major histones, mRNA in eukaryotes is polyadenylated. Nuclear poly(A) sequence has generally been assumed to be pre-messenger RNA destined to become cytoplasmic mRNA (10). Although poly(A)$^+$ RNA was initially thought to be limited to eukaryotes, it has since been found in prokaryotes as some bacterial mRNAs have poly(A) tails (45). Omega-n now shows that polyadenylation is not limited to cytoplasmic RNA, and suggests that there may be more poly(A)$^+$ RNAs that spend their lives in the nucleus.

Polyadenylation is a conserved feature of omega-n; the *D. hydei* (60 million years diverged from *D. melanogaster*) omega-n transcript is also poly(A)$^+$ (22), suggesting that polyadenylation of this nuclear RNA has a function. The polyadenylation signals associated with omega-n are quite typical of those used for cytoplasmic RNAs, suggesting that omega-n utilizes the same polyadenylation machinery. Although most polyadenylated RNAs have a perfect hexanucleotide sequence (AAUAAA) as a polyadenylation signal, variations in these signals do occur in nature and are differentially tolerated. The most common change involves an A to a U at position 2 to give AUUAAA (45). Sequences differing in more than two nucleotides from the consensus are often involved in alternative polyadenylation. Omega-n RNA appears to utilize both typical and atypical polyadenylation signals. It is of interest to note that one of the candidate atypical signals, CAUAUA, is also found upstream of a known poly(A) site in the Drosophila muscle specific protein mp20. The RNA generated by alternative termination at this site, although expressed at all stages of development, is much less prevalent than that generated by use of a more 3′ polyadenylation signal in Drosophila embryos, pupae, and adults (2). The omega-n RNA that may derive from the CAUAUA also appears to be one of the less abundant forms in the cultured cells used in these studies.

Although the functions of poly(A) tails are not completely understood, the evidence suggests that there may be multiple functions, depending on the RNA molecule that is polyadenylated (1). In one early study it was suggested that the poly(A) tail distinguished RNA molecules destined for transport to the cytoplasm (13). Omega-n and the recently discovered *Xist* RNA from the mammalian X chromosome (8, 9) now provide two examples of polyadenylated RNAs that are not transported to the cytoplasm. If polyadenylation has any effect on export from the nucleus, as suggested by experiments showing an association between removal of the last intron, polyadenylation, and export (23, 33, 34, 46), this effect must be overridden in the cases of omega-n and *Xist*. As with omega-n, polyadenylation is a conserved feature of *Xist*, having been found on both the human and mouse transcripts. This conservation suggests that, for these genes at least, polyadenylation has a function within the nucleus. For cytoplasmic RNA there is now evidence that polyadenylation can affect the efficiency of translation (24, 32) and the stability of cytoplasmic transcripts (1, 26). Although nuclear RNA is not translated, it seems probable that the poly(A) tail may be important for its stabilization. It should be noted, however, that mechanisms of RNA turnover in the nucleus need not be identical to those functioning in the cytoplasm. At least for omega-n, the majority of the turnover is sensitive to actinomycin D in a manner not observed for most cytoplasmic mRNAs.

In recent years RNA has been shown to have an increasing number of roles in the cell. Although omega-n does not fall within the generally held categories of RNA, it may represent the first of a group of RNAs with unexpected nuclear functions. Included within this group could be the mammalian *Xist* transcript. In contrast to the snRNAs which are very stable (3), levels of omega-n change rapidly in response to changes in cellular conditions. Our studies show that these changes are mediated at both transcription and turnover and suggest a new complexity in the metabolism of RNAs within the nucleus.

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