Degradation of hsp70 and Other mRNAs in Drosophila via the 5′–3′ Pathway and Its Regulation by Heat Shock

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Two general pathways of mRNA decay have been characterized in yeast. Both start with deadenylation. The major pathway then proceeds via cap hydrolysis and 5′-exonucleolytic degradation whereas the minor pathway consists of 3′-exonucleolytic decay followed by hydrolysis of the remaining cap structure. In higher eukaryotes, these pathways of mRNA decay are believed to be conserved but have not been well characterized. We have investigated the decay of the hsp70 mRNA in Drosophila Schneider cells. As shown by the use of reporter constructs, rapid deadenylation of this mRNA is directed by its 3′-untranslated region. The main deadenylase is the CCR4-NOT complex; the PAN nuclease makes a lesser contribution. Heat shock prevents deadenylation not only of the hsp70 but also of bulk mRNA. A completely deadenylated capped hsp70 mRNA decay intermediate accumulates transiently and is degraded via cap hydrolysis and 5′-deacyl. Thus, decapping is a slow step in the degradation pathway. Cap hydrolysis is also inhibited during heat shock. Degradation of reporter RNAs from the 3′-end became detectable only upon inhibition of 5′-deacyl and thus represents a minor decay pathway. Because two reporter RNAs and at least two endogenous mRNAs were degraded primarily from the 5′-end with cap hydrolysis as a slow step, this pathway appears to be of general importance for mRNA decay in Drosophila.

A characteristic feature of mRNA is its rapid turnover, permitting a continuous qualitative and quantitative adjustment of protein synthesis according to physiological needs. The pathways of eukaryotic mRNA degradation have been characterized mostly in Saccharomyces cerevisiae (1, 2). The decay of all mRNAs examined in these cells starts with deadenylation, i.e. exonucleolytic shortening of the poly(A) tail. The second step of decay does not occur until the poly(A) tail has been shortened to about a dozen nucleotides (3). In the major pathway, this second step is hydrolysis of the 5′-cap (4–6). Cap hydrolysis results in free m7GDP and a 5′-monophosphate left on the mRNA and is catalyzed by the Dcp2p subunit of the Dcp1p-Dcp2p heterodimer (7, 8). In vivo, the activity of the decapping enzyme is enhanced by several other proteins (8). The decapped RNA is degraded by the 5′-exonuclease Xrn1p (4, 5). In a minor pathway, the deadenylated RNA is degraded from the 3′-end by the exosome (9) before the remaining oligonucleotide is decapped by the enzyme Dcs1p, liberating m7GMP (10, 11).

The deadenylation-dependent decapping pathway of mRNA decay is in a fundamental competition with translation (8). Two proteins required for cap hydrolysis in vivo, Pat1p and the RNA helicase Dhh1p, act by shifting the balance in favor of cap hydrolysis at the expense of translation (12). Because the poly(A) tail is involved in translation initiation via the poly(A)-binding protein, deadenylation can be seen as promoting decapping and decay by removing the mRNA from the translated pool (13). In fact, the inhibitor of decapping is the poly(A)-binding protein, not the poly(A) tail per se (8, 14).

In animal cells, the decay of most mRNAs analyzed is also initiated by deadenylation (15–17). Subsequent decay of the deadenylated message is thought to proceed via the two pathways analyzed in yeast, mostly because the relevant proteins are conserved, but experimental data on the later steps of mRNA decay are relatively scarce. Based mainly on in vitro evidence, several groups have argued that the exosomal 3′-deacyl pathway may be the predominant mode of decay of unstable mammalian mRNAs (10, 18, 19). In vivo studies have supported a role of the 3′–5′ pathway (20–23), but evidence for the existence of the 5′–3′ pathway, sometimes acting on the same RNAs, has also been provided (17, 21–25). Conservation of the 5′–3′ decay pathway is also supported by the co-localization of the responsible proteins in distinct cytoplasmic structures, P bodies or GW bodies, in both yeast and mammalian cells (26). Furthermore, microRNA (miRNA)4-induced mRNA destabilization in Drosophila Schneider cells requires Dcp1p-Dcp2-dependent decapping (27). However, the relative contributions of the two decay pathways to overall mRNA decay remain unknown, and decay intermediates have been analyzed only to a limited extent.

Decaylation is the rate-limiting step in the decay of most mRNAs. Several arguments support this view. First, decaylation occurs by continuous shortening of the poly(A) tail throughout the lifetime of the RNA; the process can be easily followed in vivo even for unstable RNAs. However, once decaylation has occurred, the remaining oligonucleotide is degraded at a rate that is much faster than the rate of translation (28).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S3.

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4 The abbreviations used are: miRNA, microRNA; UTR, untranslated region; ARE, AU-rich element; dsRNA, double-stranded RNA; Ab, antibody; nt, nucleotide; RNP, ribonucleoprotein.

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enzyme, the homodimeric PARN (43–45), is conserved in most
mammals and in *Drosophila* is the CCR4-NOT complex (17, 36–38). A second universally conserved poly(A)-degrading enzyme, catalyzing a rapid initial shortening of the poly(A) tail (17, 42). A third poly(A)-degrading enzyme, the homodimeric PARN (43–45), is conserved in most eukaryotes, but not in yeast and in *Drosophila*. PARN has been implicated in the decay of several unstable RNAs (21, 46), but its general role in mRNA turnover remains poorly defined (17).

RNA elements promoting rapid deadenylation and decay are often located in the 3′-UTR, and the so-called AU-rich elements (AREs) are the best-studied class among them (28). Destabilizing sequences are bound by specific proteins, which can directly recruit deadenylases and other decay enzymes (21, 25, 47–50). Recently, miRNAs have also been found to be able to induce mRNA deadenylation (27, 51–53).

The *hsp70* mRNA of *Drosophila* is very unstable at normal growth temperatures with a functional half-life of 15–30 min suggested by indirect measurements (54). Rapid degradation of the *hsp70* message requires its 3′-UTR (55). Upon heat shock, the mRNA is stabilized at least 10-fold (54). This contributes to the rapid and massive (more than 1000-fold (56)) induction of *Hsp70* protein synthesis. During recovery from heat shock, the normal instability of the message is restored, and production of *Hsp70* protein ceases (54, 55). The first step in *hsp70* mRNA degradation during recovery is its deadenylation catalyzed by the CCR4-NOT complex (38, 57). An unusual, transient but pronounced accumulation of a completely deadenylated RNA species is also observed both during heat shock and recovery. Two observations suggest that deadenylation is inhibited by heat shock: First, during heat shock, the fraction of polyadenylated *hsp70* RNA increases with increasing temperature. Second, deadenylation is faster after a mild heat shock than after a severe heat shock, and deadenylation during recovery from a severe heat shock is faster if the cells have been preconditioned by a mild heat shock (57). However, the temperature dependence of deadenylation and its dependence on 3′-UTR sequences have not been tested directly.

We have examined the decay pathway of the *hsp70* mRNA in *Drosophila* Schneider cells. We find that this RNA is degraded via rapid deadenylation controlled by its 3′-UTR, followed by slow cap hydrolysis and 5′-decay. Exosome-dependent 3′-decay plays a minor role in the degradation of the *hsp70* mRNA. Both deadenylation and decapping are inhibited during heat shock. The 5′-decay pathway is also important for the degradation of several other mRNAs examined in Schneider cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** *Drosophila melanogaster* Schneider 2 cells were grown as semi-adherent layers at 25 °C in Schneider’s Insect Medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Biochrom) and 1% antibiotic/antimycotic mix (Invitrogen). Stably transformed cell lines were generated by calcium phosphate transfection and hygromycin B selection according to procedures suggested by Invitrogen (*Drosophila* Expression System Vector Version C handbook) and grown in medium containing 300 μg/ml hygromycin B. Expression of reporter gene constructs was induced at 25 °C by addition of 500 μM CuSO₄. Transcription was stopped by addition of 5 μg/ml actinomycin D (Sigma).

Heat shock was performed by incubation of cells in a temperature-controlled water bath at the temperature indicated. For induction of endogenous *hsp70* or *hsp83* RNA and analysis of their decay during subsequent recovery, heat shock was set at 35.5 or 36 °C, followed by recovery at 25 °C. For the analysis of RNAs during heat shock, 36 or 37 °C were used. No significant differences were observed between these conditions. It should be noted that the metallothionein promoter driving the reporter constructs (see below) is nearly inactive under heat shock conditions. Thus, when actinomycin D was added some time after heat shock, the distribution of RNA decay intermediates at the time of actinomycin D addition reflected a period of net decay preceding the addition of the drug. Apart from that, no difference in RNA decay was seen when actinomycin D was added at the time of heat shock or 30 min later.

**Plasmids—** The reporter plasmids were constructed as follows: The mutated RNP domain of bovine PABPN1 (Met161–Thr258 with the mutations Y175A, K213Q, and F215A) (58) was amplified by PCR with the primers GGTACCATGTCCATTGAGGAGAAGAT introducing a KpnI restriction site (underlined) at the beginning of the open reading frame and GTCGACTGTTGTGCTGATGCCTGGT introducing a SalI site (underlined) at the end. The 3′-UTR and additional downstream sequences of the distal *hsp70* gene of the 87A7 locus (gene Ah, GenBank™ accession number CG18743 nucleotides 1–243, 1 being the first nucleotide behind the stop codon) were amplified from genomic *D. melanogaster* DNA (strain Oregon 1) with primers *Hsp70* Sal (5′-GTCGACTAAAGCCAAAAAGA-3′), binding at the border of the open reading frame and the 3′-UTR and containing the naturally occurring SalI site immediately 5′ of the stop codon, and *Hsp70* DSE (5′-GAATTCAGACTTCAAAATGGTTTCTGCT-3′), introducing a new EcoRI site at the 3′-end. The 3′-UTR plus downstream sequences of the *Drosophila adh* gene (GenBank™ accession number X78384, nucleotides 2926–3226) were amplified from a plasmid clone (a gift of Saverio Brogna) with the primers ADH sense Sal (5′-GTCGACTAAAGAAATGATAATCCCA-3′), which introduces a SalI restriction site followed by a stop codon.
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at the 5'-end, and ADH DSE (5'-GAATTCTTTTTGACTCGGTTTC-3'), which introduces an EcoRI restriction site at the 3'-end of the PCR product. All fragments were cloned into a pBS-SK vector (Stratagene) opened with Smal. The RNP fragment was cut out with KpnI/Sall, purified, and ligated into the KpnI/Sall-opened plasmids already containing the HSP70 and ADH 3'-UTRs. The Sall site introduces the additional sequence Val-Thr at the C terminus of the RNP domain. The complete RNP-UTR fragments were then cut out with KpnI and EcoRI and ligated into the KpnI/EcoRI-opened pMT/V5 expression vector (version A, Invitrogen), from which a BclI fragment containing the SV40 poly(A) signal had been deleted. Point mutations in the ARE sequences of the HSP70 reporter plasmid were introduced in two sequential reactions via the QuikChange site-directed mutagenesis method (Stratagene). The sequence was verified after each round of mutagenesis.

RNA Interference—dsRNA was prepared by enzymatic synthesis (Ambion MEGAscript® T7 Kit) from DNA templates obtained by PCR. PCR templates were either plasmid clones (Pan2 and Xrn1, the latter a gift of Sarah Newbury) or oligo(dT)-primed cDNA derived from S2 cell RNA (all others). Primer pairs are listed in supplemental Table S1. 45 µg of dsRNA was placed into a 60-mm cell culture dish. Logarithmically growing cells were diluted with serum-free medium to 1 × 10^6/ml (final serum concentration 1.5% or less), 3 ml were added per dish, incubated for 30 min at 25 °C and then mixed with 6 ml of medium containing 10% serum. After 4 days, cells were split into 1-ml aliquots. When the dsRNA treatment reduced the growth rate, two batches of knock-down cells were combined and split into 2-ml aliquots. If knock-down efficiency was to be confirmed by Western blotting, protein was isolated from one aliquot: Cells were pelleted, resuspended in 75 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 8% glycerol) and frozen in liquid nitrogen and thawed on ice. Total RNA was isolated from all other aliquots according to the TRiZol® protocol (Invitrogen). Pelleted RNA was dried at room temperature and dissolved in water by incubation at 65 °C for 20–30 min. Concentrations were determined by spectrophotometry. If no antibody was available to confirm the knock-down, semi-quantitative RT-PCR was done as follows: 1.5 µg of total RNA from knock-down and control cells was reverse-transcribed with 20 pmol dT12 primer and 150 units of murine leukemia virus reverse transcriptase (Promega) in a 25-µl reaction as recommended by the supplier. For the subsequent PCR, 1 µl of this reaction was used as template in a total volume of 80 µl of 1× green GoTaq® Flexi buffer (Promega) containing 1.5 mM MgCl2, 200 µM dNTPs, 1 µM primer (each), and 0.5 units of GoTaq® DNA polymerase (Promega). Primers are listed in supplemental Table S2. After 2 min at 94 °C, cycles consisted of 15 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C. After 29, 31 and 33 cycles, aliquots were taken and analyzed on a 1% agarose gel. Cytochrome c cDNA was amplified as a control.

Northern Blots—Probes directed against the PABPN1 RNP domain and the hsp70 3'-UTR were synthesized by transcription of linearized plasmids similar to those described above. All other probes were generated by transcription of templates obtained by RT-PCR (supplemental Table S3). T7 RNA polymerase (Promega) was used in the presence of 10–40 µCi of [α-32P]UTP (Amersham Biosciences) as recommended by the supplier. Total RNA (between 1 and 2.5 µg depending on the experiment) was electrophoretically separated on a 5% polyacrylamide-8.3% urea gel and transferred to a nylon membrane (Hybond-N, Amersham Biosciences) by semidy electroblotting in 0.5× Tris borate EDTA. After UV irradiation, hybridization and washing were carried out as described (59), and the blots were exposed to a phosphorimagier screen.

Cap Analysis—Total RNA (10 or 15 µg depending on the experiment) was denatured at 95 °C for 3 min and placed on ice. Depending on the amount of RNA used, 1 or 1.5 units of Terminator™ 5'-phosphate-dependent exonuclease (Epicentre) was added, the volume was adjusted to 20 or 30 µl, and the mixture was incubated at 30 °C for 2 h under conditions specified by the supplier. The volume was adjusted to 200 µl with water, 10 µg of glycogen was added, the RNA was purified by phenol/chloroform extraction and ethanol precipitation and dissolved either in 10 µl of 1× EM (for subsequent RNAse H digestion) or in formamide-loading buffer.

RNase H Digestion—RNase H digestion was performed as described (57) with the following modifications: 10 µg of RNA was used for each digestion. Oligonucleotide 1 (5'-GTCCA-GAGTACGCGCAAATCTCTCGGCC-3') was used for the hsp70 mRNA, oligonucleotide 2 (5'-TTAATCGACCTTCTCATGTGGGAAAGC-3') for the hsp83 mRNA, and dT12 instead of dT15 for removal of the poly(A) tail. RNase H was from Promega, and digestion was for 45 min at 37 °C. 10 µg of glycogen was added, RNA was pelleted by ammonium acetate/ethanol precipitation, washed with 70% ethanol, air-dried, and dissolved in formamide-loading buffer.

Mapping of the hsp70 Ab Polyadenylation Site—3 µg of total RNA from stable cell lines in which the transcription of the RNP-HSP70 3'-UTR or the RNP-HSP70 3'-UTR ARE constructs had been induced was used for RT-PCR amplification of the 3'-UTR and poly(A) tail as described earlier (38). Amplification was carried out with the Hsp70 Sal primer (see above) and the oligo(dT) anchor primer. The PCR products were purified and sequenced with the Hsp70 Sal primer.

Bulk Poly(A) Analysis—The length of bulk poly(A) was analyzed essentially as described previously (38). As a control for the specificity of the RNAse digestion, in vitro-synthesized L3pre RNA with an A50 tail (60) was treated in the same way.

RNA was separated on a 10% polyacrylamide-urea gel. Gels were analyzed by phosphorimaging with the help of the Image-Quant program.

Western Blots—15 µl of S2 extract was separated on a 6 or 10% SDS-polyacrylamide gel and blotted onto polyvinylidene fluoride membrane. Blots were blocked in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20, 2.5% bovine serum albumin. The same buffer was also used for incubation with antisera and washing. Affinity-purified anti-CAF1 antibody (38) was used at 1:1000 dilution, anti-XRN1 antibody (a gift of Sarah Newbury) was used at a 1:2500 dilution. As loading control, α-tubulin antibody (Sigma) was used at a 1:5000 dilution. Antibodies were detected by peroxidase-conjugated swine anti-rabbit or rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark) and chemiluminescence staining (SuperSignal kit, Pierce).
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**RESULTS**

The hsp70 3′-UTR Is Sufficient to Mediate Rapid Deadenylation and Regulation by Heat Shock—To analyze the influence of the hsp70 3′-UTR on deadenylation and its regulation by temperature, we constructed two reporter genes. Both contained the same open reading frame, the RNP domain of the bovine nuclear poly(A)-binding protein carrying three point mutations. The wild-type RNP domain by itself has a very low affinity for poly(A), which is further reduced by the mutations (58). Thus, the protein is expected not to affect poly(A) tail metabolism, and this is borne out by the experiments presented below. The open reading frame was fused to the 3′-UTR and downstream genomic sequence containing the 3′-processing signal of either the adh or hsp70 gene. Reporter constructs were put under the control of the Drosophila metallothionein promoter allowing induction of transcription by copper sulfate independently of growth temperature. Drosophila S2 tissue culture cells were stably transformed with the two constructs (RNP-ADH 3′-UTR and RNP-HSP70 3′-UTR constructs, respectively; Fig. 1A). The RNAs will be referred to as RNP-ADH and RNP-HSP70.

Deadenylation and decay of the reporter RNAs were determined by Northern blotting of samples obtained at different times after inhibition of transcription by actinomycin D. Treatment of steady-state RNA with RNase H and oligo(dT) provided a marker for deadenylated RNA (compare lanes 1 and 2 and lanes 8 and 9 in Fig. 1B; right panel in Fig. 1C). The sizes of the deadenylated RNAs were consistent with the use of the natural polyadenylation sites of the adh and hsp70 genes (see also Fig. 2). The 7SL RNA served as a loading control. In pulse-chase experiments, actinomycin D was added 45 min after the induction of transcription. In the case of the RNP-ADH construct, this short pulse of transcription resulted in a homogeneous population of RNAs carrying poly(A) tails of 120–200 nt, which were then shortened over time (Fig. 1B, left panel, lanes 3–7). Two distinct decay intermediates, one carrying an oligo(A) tail of about 20 nt, the other completely deadenylated, became first visible 60–90 min after transcription shut-off. Substantial amounts of poly(A) tails of up to 110 nt were left at 180 min. On the basis of the upper end of the poly(A) tail length distribution, the rates of deadenylation were estimated as 0.5–1 nt/min in several experiments. The total amount of RNP-ADH RNA did not decrease significantly over the 180 min time course. This experiment confirms the expectation that deadenylation precedes decay of the mRNA body. Transient accumulation of the intermediates further suggests that shortening of an A180 intermediate to A0 is a slow step and, surprisingly, that the step subsequent to deadenylation is also slow.

In the case of the RNP-HSP70 construct, the pulse-chase experiments revealed a very heterogeneous population at the time of transcription shut-off with poly(A) tail lengths ranging from 0 to 190 nt. Similarity of the RNA pattern after the short transcription pulse to that of steady state RNP-HSP70 RNA (Fig. 1B, left panel, compare lanes 8 and 10) suggests that this RNA was very unstable. Indeed the remaining poly(A) tails were lost rapidly, and only a poly(A)-deficient decay intermediate was left within one hour after actinomycin D addition. In
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A

| WT-UTR: | 136 CCAAGTCGTTAATTTGCGAAAAATTTGATTGTTGTTGTTGTTT |
| Mut-UTR: | ATTGTAAATTTGATTGTTGTTGTTGTTT |
| WT-UTR: | 193 GAAATAAGTCGTTAATTTGCGAAAAATTTGATTGTTGTTGTTT |
| Mut-UTR: | GCTTAATTTGATTGTTGTTGTTGTTT |

FIGURE 2. AREs are not required for the rapid deadenylation of hsp70. A, relevant part of the downstream sequences used in the RNP-HSP70 3′-UTR construct is shown. The polyadenylation signal AUUAAA is underlined. The arrowhead indicates the polyadenylation site, and downstream sequences are shown in lowercase letters (see “Experimental Procedures”; note that the first three nucleotides of the poly(A) tail could be either DNA-encoded or added post-transcriptionally). This site corresponds to the one used in the almost identical lower sequence (see “Experimental Procedures”). Heat shock (57). Thus, the 3′-UTR of the hsp70 mRNA is sufficient to control these events.

Decay time courses were also analyzed after transcription had been induced for 3 h (Fig. 1C). In this case, the distribution of the RNP-ADH RNA at t = 0 was more heterogeneous, including complete deadenylation, but had not reached the steady state (compare with Fig. 7B, luciferase control). The patterns of deadenylation and decay for both RNAs confirmed what had been seen after a short pulse of transcription, and rates of deadenylation were similar to those reported above. Half-lives of the RNP-ADH and RNP-HSP70 RNAs were estimated as 200 and 50 min, respectively.

Decay experiments were also performed under heat shock conditions (36 or 37 °C; see “Experimental Procedures” for details) after long or short pulses of transcription at 25 °C. Depending on the experiment, actinomycin D was added either at the beginning of heat shock or 30 min later (see “Experimental Procedures”). Heat shock inhibited deadenylation of both reporter RNAs very strongly (Fig. 1, B and C). The oligoadenylation intermediate of the RNP-ADH RNA was not seen under these conditions. Consistent with a dependence of mRNA body decay on prior deadenylation, the total amount of either reporter RNA did not decrease significantly during the 180 min time course. Because the abundance of the deadenylated decay intermediate did not decrease either, a step subsequent to deadenylation was also inhibited during heat shock. As demonstrated below, this step is cap hydrolysis.

AUUUA Sequences Are Dispensable for Rapid Deadenylation—D. melanogaster has five hsp70 genes in two clusters. Cluster A is formed by genes Aa and Ab. The 3′-UTR of Ab used in this study is nearly identical to that of Aa; both contain three AUUUA sequences, which are known mRNA destabilizing elements in mammalian cells. The last AUUUA overlaps with the beginning of the poly(A) tail (Fig. 2A). The 3′-UTR sequences of the three hsp70 genes in cluster B at locus 87B diverge from those in cluster A, but at least two of them are essentially identical with each other and contain five AUUUA sequences, four of which form two overlapping pairs, particularly efficient decay-promoting elements in mammalian cells (31, 32). Single or double point mutations were introduced simultaneously into all three AUUUA motifs of the RNP-HSP70 construct (Fig. 2A), and stably transformed cell lines were generated. Although the mutations were close to the polyadenylation signal, they had no effect on the site or efficiency of 3′-processing (Fig. 2). Surprisingly, the combined mutations did not affect the deadenylation rates, which were ~3 nt/min for either RNA. Overall decay rates were also indistinguishable (Fig. 2B). Thus, the AUUUA motifs are irrelevant for the rapid deadenylation of the hsp70 mRNA, and the destabilizing elements remain to be identified.

Bulk mRNA Deadenylation Is Inhibited During Heat Shock—The behavior of bulk poly(A) during an actinomycin D chase was tested by 3′-end-labeling of the RNA, digestion of the non-poly(A) portions with a combination of RNases A and T1, and analysis of the remaining labeled poly(A) tail population on denaturing gels. At the normal growth temperature, the upper end of the length distribution shrunk from about 250 to about 150 nts over the 5-h time course. In contrast, the length distribution remained stable over the same time course under heat shock conditions (Fig. 3). Thus, heat shock leads to an arrest of bulk mRNA deadenylation.

The PAN2-PAN3 Enzyme Makes a Minor Contribution to Deadenylation—The CCR4-NOT complex is the major enzyme responsible for hsp70 and bulk mRNA deadenylation in Schneider cells (38). To test for a possible involvement of the PAN2-PAN3 deadenylase, we carried out RNA interference experiments and examined the decay of the endogenous hsp70 mRNA during recovery from heat shock. Under these conditions, the decay of the hsp70 RNA accumulated during the preceding heat shock can be followed without the use of actinomycin D (38). Because the hsp70 mRNA is too long for a direct analysis of deadenylation, it was shortened by RNase H digestion in the presence of a DNA oligonucleotide hybridizing to the end of the open reading frame, and the resulting polyadenylated 3′-fragment was analyzed on high-resolution Northern blots (57). In comparison to control cells treated with dou-
ble-stranded luciferase RNA, knock-down of the CAF1 subunit of the CCR4-H18528 NOT complex retarded deadenylation, as reported before (38). However, simultaneous knock-down of both PAN2 and CAF1 led to a more severe inhibition of deadenylation (Fig. 4A and quantitation in supplemental Fig. S1). The knock-downs of PAN2 and CAF1 were verified by semi-quantitative RT-PCR and Western blotting, respectively (Fig. 4B). In several experiments, the additional inhibition of deadenylation was seen only when the PAN2 knock-down was particularly efficient, and knock-down of PAN2 by itself had no effect (supplemental Fig. S2). We conclude that PAN contributes to hsp70 deadenylation but is less important than the CCR4-H18528 NOT complex.

The Accumulating Poly(A)-deficient Intermediate of hsp70 mRNA Degradation Is Capped—Degradation of the hsp70 mRNA and both reporter RNAs proceeds via transiently accumulating poly(A)-deficient intermediates, indicating that a step subsequent to deadenylation is slow (57) (Fig. 1). To find out whether this intermediate is capped, we made use of a 5′-exonuclease that only digests RNA with a 5′-phosphate, which is the expected product of cap hydrolysis by DCP2; capped RNA is resistant to the exonuclease. Because the 5′-exonuclease is Mg2+-dependent, incubation in the presence of EDTA was used as a control for nonspecific RNase activity. For the purpose of the experiment, cells recovering after heat shock were used as a source of endogenous hsp70 mRNA consisting of both polyadenylated and deadenylated species. Polyadenylated RNA is expected to be capped and thus provides a convenient 5′-exonuclease-resistant internal control. RNase H shortening of the hsp70 RNA remaining after 5′-exonuclease digestion allowed the discrimination of polyadenylated and non-polyadenylated species.

Both polyadenylated and deadenylated hsp70 mRNA were resistant to the 5′-exonuclease (Fig. 5A; compare exonuclease digestion to the EDTA control), indicating that the deadenylated intermediate retained its cap. As additional controls, the 2,2,7-trimethyl G-cap bearing U1 snRNA was also completely resistant, whereas 5.8 S rRNA with its 5′-phosphate was almost completely degraded. A potential pitfall of this experiment is that the exonuclease might have attacked the hsp70 mRNA without reaching the 3′-UTR sequences finally detected on the Northern blots, being blocked either by the sheer size of the RNA or secondary structures. However, very similar results were obtained with the RNP-HSP70 reporter RNA both at 25 and 37 °C (Fig. 5B). Compared with the hsp70 mRNA, this RNA...
is less than one quarter the size and carries a completely different 5′-UTR and coding sequence; its resistance to the 5′-exonuclease confirms that the deadenylated intermediates are capped. The experiments thus lead to the conclusion that decapping either does not play any role in the decay of the RNAs examined or, alternatively, that cap hydrolysis represents a slow step.

The hsp70 mRNA Is Degraded via Cap Hydrolysis and 5′-Decay—RNA interference was used to determine by which pathway the deadenylated intermediate of the endogenous hsp70 mRNA is degraded during recovery from heat shock. The knock-down of the decapping enzyme DCP2 resulted in a pronounced stabilization of the deadenylated intermediate compared with the control cells treated with double-stranded luciferase RNA (Fig. 6, A and C). The knock-down was verified by semi-quantitative RT-PCR (Fig. 6B). The result clearly demonstrates that DCP2-dependent cap hydrolysis is the predominant second step in the decay of the hsp70 mRNA. Because the deadenylated substrate for cap hydrolysis accumulates transiently even with the normal level of DCP2 protein (Figs. 4A and 6A, luciferase controls), the results also identify decapping as a slow step in hsp70 mRNA decay.

The knock-down of DCP2 also retarded the degradation of the deadenylated decay intermediate of the RNP-HSP70 RNA and led to a 2-fold decrease in overall decay rate; thus, cap hydrolysis is the second step in the decay of this RNA as well (Fig. 7A). Because the deadenylated intermediate is stabilized during heat shock (Fig. 1C) and is fully capped (Fig. 5B), the experiment also shows that cap hydrolysis is inhibited during heat shock. In contrast to the stable RNP-ADH RNA, stabilization by the DCP2 knock-down was not significant, presumably because deadenylation was rate-limiting. However, two details suggest that this RNA is also a DCP2 substrate. First, a shorter RNA species, which was barely visible under normal conditions, accumulated noticeably upon DCP2 knock-down (Fig. 7B, arrow). This RNA probably represents an intermediate of degradation from the 3′-end (see below). Second, the oligoadénylated decay intermediate was more heavily populated under conditions of DCP2 knock-down (Fig. 7B). This was observed at all time points of two independent experiments (quantitation not shown) and suggests that this species is a substrate for decapping.

Knock-down of the 5′-exonuclease XRN1 (Pacman in Drosophila) (61), verified by Western blotting (Fig. 6B), led to a modest but reproducible stabilization of a full-length deadenylation decay intermediate of the hsp70 RNA (Fig. 6, A and C). Thus, XRN1 degrades the RNA after decapping, as expected. That the accumulation is moderate is not surprising as decapping either does not play any role in the decay of the RNAs examined or, alternatively, that cap hydrolysis represents a slow step. Also, the gel does not resolve the uncapped intermediate expected to pile up upon knock-down of XRN1.
from the capped intermediate, which is heavily populated anyway.

Exosome-dependent 3'-Decay Plays a Minor Role in mRNA Decay—Interestingly, the analysis of the RNP-ADH and RNP-HSP70 RNAs upon Dcp2 knock-down revealed the appearance of additional shorter RNA species (Fig. 7, arrows). Because these RNAs appeared when 5'-decay was inhibited, they could be generated either by endonucleolytic cleavage or by 3'-decay pausing at specific sites. The yeast Ski3 protein is specifically required for the cytoplasmic function of the exosome (9). When the Drosophila SKI3 protein was knocked down in combination with DCP2, the abundance of the shorter decay intermediates of the RNP-HSP70 RNA was strongly decreased (Fig. 8A). Thus, 3'-degradation by the exosome appears to be a second pathway for the degradation of the RNP-HSP70 mRNA. RT-PCR analysis (Fig. 8B) suggested that the DCP2 knock-down was less efficient when SKI3 was knocked-down simultaneously, and this might be an alternative explanation for the disappearance of the short decay intermediates. This is considered unlikely for two reasons: First, the rate of decay of the deadenylated intermediate was not noticeably different between the single and double knock-down. Second, the normal variation in the efficiencies of DCP2 single knock-downs never led to the disappearance of the shorter intermediates. Because the intermediates reflecting exosomal degradation of the RNAs appeared only upon inhibition of 5'-decay, the contribution of 3'-decay to the degradation of the reporter RNAs under normal conditions must be minor. The same is true for the endogenous hsp70 RNA as seen from the very strong stabilization by a Dcp2 knock-down (Fig. 5). Moreover, knock-down of SKI3 by itself had no detectable effect on the stabilities of the endogenous hsp70 RNA and of either reporter RNA (supplemental Fig. S3).

5'-Decay Is an Important Pathway in the Degradation of Other mRNAs—RNA samples obtained during recovery from heat shock were analyzed by Northern blotting for the decay of the hsp83 mRNA (Fig. 9). Although synthesis of hsp83 mRNA has been reported to continue at a low level during recovery (57) and no actinomycin D was used in this experiment, the decay of RNA synthesized during heat shock can be easily observed, as shown by the gradual deadenylation. A 2-fold
Degradation of the Drosophila hsp70 mRNA

FIGURE 9. 5' decid is important for the decay of the hsp83 mRNA. S2 cells were treated with the dsRNAs indicated above the lanes for 4 days. Cells were heat-shocked at 35.5°C for 30 min and then incubated at 25°C to allow recovery. Total RNA was isolated at the indicated times of recovery, digested with RNase H in the presence of oligonucleotide 2 and analyzed by Northern blot with a probe directed against the 3'-UTR of hsp83 mRNA and subsequently with a 7SL RNA probe as a loading control. Lane 2, RNA from heat-shocked cells that had not received an RNAi treatment was digested with oligonucleotide 2 and oligo(dT). Note that the HSP83 gene contains an intron. While our experiment does not distinguish between spliced and unspliced RNA, the induction temperature used here would not be expected to inhibit splicing (79).

stronger accumulation of the deadenylated decay intermediate upon knock-down of DCP2 clearly indicates that the 5'-decay pathway makes a significant contribution to the decay of the hsp83 mRNA. Because the deadenylated intermediate was also visible in control cells, deamidation is a relatively slow step in the normal decay pathway as well. In preliminary experiments, the D-myc mRNA was also stabilized by a DCP2 knock-down (data not shown). Overall, these data suggest that deamidation-dependent decapping followed by 5'-hydrolysis is a major pathway of mRNA decay in Drosophila Schneider cells.

DISCUSSION

In this report, we have analyzed the decay pathway of the hsp70 mRNA of D. melanogaster. Rapid degradation of this RNA under normal growth conditions is initiated by deamidation, which is followed by decapping and degradation from the 5'-end. This corresponds to the major mRNA decay pathway in S. cerevisiae. Not surprisingly, the rapid deamidation of the hsp70 mRNA under normal growth conditions is directed by its 3'-UTR. However, mutagenesis of the three AUUUA elements in the 3'-UTR had no effect on the rate of deamidation or overall decay. Thus, sequences identical with classical mammalian AREs do not appear to function as destabilizing elements in Drosophila.

The main deamidase active in the degradation of the hsp70 mRNA is the CCR4-NOT complex (38). We now find that the PAN2-PAN3 complex also contributes to deamidation. The patterns of deamidation intermediates are not sufficiently distinct to permit any conclusions regarding a particular order in which the two deamidases act. The decay pattern of the RNP-ADH RNA shows that terminal deamidation, going from an A20 intermediate to complete loss of the poly(A) tail, can be kinetically distinct. Whether this reflects the activities of two different enzymes or a change in the substrate, e.g. dissociation of the last molecule of cytoplasmic poly(A)-binding protein, and why the A20 intermediate is not seen with all mRNAs remains to be investigated.

An unusual feature of mRNA decay in Schneider cells is the low rate of cap hydrolysis, which leads to a visible accumulation of completely deamidated decay intermediates of all RNAs examined. This is in contrast to the situation in yeast, where the rate of cap hydrolysis is fast compared with the rate of terminal deamidation; thus oligoadenylated intermediates are decapped and degraded, and complete deamidation usually does not occur (3, 5). In mammalian cells, levels of completely deamidated RNAs are also low (17, 24). Although the rate of deamidation may have some influence on the extent to which the deamidated intermediates accumulate, the phenomenon was observed for the rapidly deamidated hsp70 and RNP-HSP70 RNAs (e.g. Figs. 1, 4), the hsp83 RNA (Fig. 9) and the relatively stable RNP-ADH RNA (Fig. 7). The deamidated intermediates were present in the steady-state populations of all these RNAs in cells in the absence of heat-shock or actinomycin D treatment; therefore, cap hydrolysis is slow under normal conditions.

Although decapping limits the physical half-life of the hsp70 mRNA, the functional half-life of this message is expected to be limited by deamidation, as the poly(A) tail is important for translation. Unfortunately, we have been unable to test this directly by checking the polysome association of polyadenylated and deamidated hsp70 mRNA; RNase activity in the extracts was too high on the time-scale required for the experiments. However, Dellavalle et al. (57) have reported that, upon sustained heat stress, all hsp70 mRNA accumulates as a deamidated species, and this RNA is apparently no longer translated. Izaurralde and co-workers (27) have also reported experiments suggesting that deamidated mRNA is not translated in Schneider cells.

Cap hydrolysis is slow under normal conditions and can be further retarded by DCP2 knock-down. This provides a very sensitive assay for any contribution of additional decay pathways. Indeed, intermediates reflecting exosomal 3'-decay of reporter constructs were detected. Because these intermediates became visible only upon knock-down of DCP2, the contribution of 3'-decay must be minor. This is supported by the very strong stabilizing influence of the DCP2 knock-down on the endogenous hsp70 mRNA. During recovery from heat shock, stabilization was roughly 10-fold (Fig. 6). Thus, the 3'-decay pathway probably contributes 10% or less to the overall decay of this RNA.

The 5'-decay pathway was found to be important not just for the hsp70 mRNA and the two reporter RNAs but also for the hsp83 and, very likely, the D-myc mRNAs. This is in good agreement with the importance of this pathway in miRNA-induced mRNA decay in Schneider cells (27). Together, these data suggest that, as in yeast, deamidation-dependent decapping may be the primary pathway for mRNA decay in Schneider cells.

Deamidation of the hsp70 mRNA is inhibited during heat shock, not only in Drosophila but also in mammalian cells (62, 63). This contributes to the rapid induction of HSP70 protein synthesis when it is required. An inhibition of deamidation during heat shock has also been reported for other unstable mRNAs in mammalian cells (64–66) and in yeast (67). Indeed, we find that bulk mRNA deamidation is inhibited. Inhibition of deamidation and decay of a number of mRNAs has also
have been observed under several other stress conditions, again both in mammalian cells (68–71) and in yeast (67). Stabilization of mRNA under stress conditions may serve to facilitate the recovery from stress (67).

How does cellular stress lead to a general inhibition of mRNA deadenylation? The regulatory mechanism must be post-translational, since both the inhibition of deadenylation during heat shock and the reactivation of deadenylation during recovery are resistant to actinomycin D and cycloheximide (57) (this work). In mammalian cells, the double-stranded RNA-dependent protein kinase PKR is essential for the stabilization of hsp70 mRNA during heat shock (63), but the relevant substrates are unknown. At least three mechanisms of inhibition, which are not mutually exclusive, can be considered.

First, stress might lead to the inactivation of specific mRNA binding proteins promoting deadenylation, e.g. ARE-binding proteins in mammals. For example, activation of the p38 MAP kinase pathway by inflammatory stimuli leads to phosphorylation of the decay-promoting, ARE-binding protein TTP by MAPKAP kinase 2 and stabilization of ARE-containing mRNAs (72–74). Because the p38 MAP kinase is also activated by stress, this type of regulation is likely to contribute to mRNA stabilization during the stress response. However, the effect of the p38 MAP kinase pathway is limited to certain ARE-containing mRNAs and is, therefore, unlikely to account for a stabilization of bulk mRNA. For example, although UV-irradiation leads to an activation of p38 MAP kinase, UV-induced mRNA stabilization is independent of this pathway (69). Similarly, other regulatory mechanisms acting on specific mRNA binding proteins (66) probably do not account for the inhibition of bulk deadenylation.

Second, inhibition of deadenylation might be a consequence of the inhibition of translation usually associated with stress. For example, sequestration of mRNA in stress granules (75, 76) might protect them from deadenylating enzymes. Most likely, this is not a sufficient explanation, because the hsp70 mRNA is translated during heat shock, and it has been experimentally verified that this RNA is not found in stress granules (75). In contrast, the RNP-HSP70 reporter construct is expected to behave like bulk mRNA and cease translation during heat shock as it does not contain the hsp70 5’-UTR, which is responsible for the preferential translation of hsp70 mRNA during heat shock (77, 78). Nevertheless, the reporter behaves like the translated endogenous hsp70 mRNA with respect to deadenylation. Thus, translation does not play an important role in the regulation of deadenylation during heat shock. Similar conclusions have been reached regarding the inhibition of deadenylation during osmotic stress in yeast (67).

Third, the deadenylase activity itself may be inhibited during heat shock, either by a direct regulation of the enzyme or perhaps by a general change in the properties of the poly(A)-PABPC complex which is the substrate for poly(A) degradation. These possibilities remain to be investigated.

Although cap hydrolysis is slow under normal growth conditions in Drosophila S2 cells, it seems to be further inhibited during heat shock, as suggested by the stabilization of deadenylated decay intermediates during heat shock (57) (this work). Inhibition of decapping may also contribute to the preservation of mRNA under stress conditions. It will be interesting to find out whether this RNA can be re-adenylated during recovery from heat shock.

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