Terminal sequences do not contain the rate-limiting decay determinants of *E.coli cat* mRNA

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ABSTRACT

The mechanism of *E. coli* chloramphenicol acetyltransferase (*cat*) mRNA decay was investigated. Alteration of the 5' untranslated terminus does not appear to have an effect on the turnover rate of the mRNA. Similarly, changes at the 3' terminus of the message, including the addition of a stable stem and loop structure, do not affect the half-life of the message. The data suggest that 5' and 3' terminal untranslated sequences do not contain the rate-limiting determinants for *cat* message decay. Decay rates for various segments of the *cat* mRNA were measured and indicate that all regions of the message have similar stabilities. The current model of *cat* mRNA degradation involves a rate-limiting endonucleolytic decay event that occurs internal to the message followed by degradation of the cleavage products.

INTRODUCTION

The importance of mRNA stability in gene regulation has been demonstrated in a number of systems (1). The factors which are involved in determining the stability of messages have not been well defined, although a number of parameters have been described which can affect message turnover rate (2). One of these parameters involves the association of ribosomes with mRNA and the rate of translation. This interaction is thought to protect the message and decrease its susceptibility to nucleolytic digestion (3,4). However, some point mutations in the *lac* operator sequence which increase translation initiation decrease the half-life of the *lac* message (5). The composition of the mRNA can also affect turnover of the message. In *Saccharomyces cerevisiae* it appears that the length of an mRNA species may correlate with its decay rate (6).

Among prokaryotic messages, there are examples of sequences which have the potential of forming secondary structures that affect mRNA stability. These determinants have been found at the 3' terminus and within intercistronic regions of a message. A stem and loop structure located at the 3' end of an mRNA can stabilize upstream sequences, presumably from 3' to 5' exonucleolytic activity, as in the case of the *cry* mRNA of *Bacillus thuringiensis* (7). REP sequences, which form large stem and loop structures in intercistronic regions of some *E. coli* operons, also appear to stabilize upstream sequences (8). Although this type of structure may be necessary for stabilization of particular upstream RNA sequences, it may not be sufficient as a stabilization factor as in the case of the stem and loop structure within the *Rhodobacter capsulatus puf* operon (9). Other secondary structures, such as those which form RNase III cleavage sites, can func-

tion as decay initiation points. These structures have been found at the 3' end of mRNA as in the *λ*int message (10-12) or in intercistronic regions of the polycistronic *pnp* and *pap* containing mRNAs of *E. coli* (13,14).

In addition to secondary structures that may affect exonuclease activities or particular endonuclease cleavages, other specific regions of an mRNA molecule also appear to affect mRNA turnover. These regions of the mRNA define endonucleolytic cleavage sites that may determine rate-limiting decay events. Cleavages which occur in the 5' untranslated region of the *ompA* message are reported to be rate-limiting to message decay (15), although under certain culture growth conditions there appears to be a more labile region within the 3' end of the *ompA* mRNA (15,16). Specific endonucleolytic cleavages have also been reported in the *cat* message in *Bacillus subtilis* (17) and in the *E. coli trp* (18,19), *lac* (20) and *gal* (21) messages. The most well-studied system that actually defines the endonucleolytic cleavages by direct sequencing is the *lac* operon. Several cleavages within the *lacZY* intercistronic region as well as in the *lacY* coding sequence have been detected (22). By comparing the sequences around the cleavage sites, an A+U rich sequence was recognized that appears to be more susceptible to cleavage (22). The authors propose a model for mRNA decay that involves an initial rate-limiting endonucleolytic cleavage that occurs at any one of several sites followed by rapid decay of the cleavage products.

In this study we determined the effects of alterations in the untranslated regions of the *cat* mRNA on turnover rate. We found that the length of the 5' or 3' untranslated region or the placement of a secondary structure at the 3' end of the message did not appear to affect the decay rate. These results together with the analysis of decay rates of various domains of *cat* mRNA suggest that rate limiting decay events do not rely on specific sequences at the termini of the message.

MATERIALS AND METHODS

RNA Isolation

Cultures of *E. coli* strain MC1000 (23) harboring plasmid pACYC184 (24), pJJS310 (25) or pCD1018 were grown in M9 minimal medium with 0.2% glucose at 30°C. Five ml aliquots were withdrawn before and at various times after the addition of rifampicin to a final concentration of 200 µg/ml. RNA was isolated from the cells in the presence of diethylpyrocarbonate (26).

Detection of *cat* Specific RNA Species

Approximately 40 µg of total RNA isolated from cells containing pACYC184, pJJS310 or pCD1018 were denatured and electrophoresed on a 1.75% agarose/6% formaldehyde gel in MOPS buffer pH 7 (27). Molecular size markers were denatured TaqI pBR322 fragments which had been radiolabelled at the 3' end. After electrophoresis the RNA was capillary transferred to Zeta Probe nylon membrane (BioRad). The hybridization solution contained (final concentra-

tion): 50% formamide, 0.5 M sodium phosphate buffer pH 7.2, 1 mM EDTA, 1% BSA, and 7% SDS. The first probe was the 977 bp Sau3AI *cat* fragment from pACYC184 which was isolated and then radiolabelled by extension of random primers (28). The alternate probe was the recombinant single stranded phage DNA from M13CAT-3 (see DNA Sequencing in Materials and Methods). The chimeric single stranded DNA, which contains the template strand of the Sau3AI *cat* fragment, was extracted from its protein coat (29) and radiolabelled by extension of the probe primer (30). Wash conditions of blots were as follows: 2x SSC, 5 min at room temperature, twice; 2x SSC, 1% SDS, 30 min, 65°C, twice; and 0.1x SSC, 1% SDS, 30 min, 65°C, twice. The filters were then exposed to X-ray film which had been preflashed to an O.D._{650 nm} of 0.1.

Nuclease S1 Mapping

a. 5' End Mapping. The 5' hybridization probe was a 368 bp EcoRI fragment from pJJS310 which includes the *lac* promoter/operator and the initial 273 bp of the 977 bp Sau3AI *cat* fragment. The fragment was radiolabelled at its 5' end. Hybridization was carried out by incubation of the probe with the RNA overnight at 45°C (27). 1000 U nuclease S1 was added to the samples and incubated at room temperature for 2 hours. The phenol: chloroform (1:1) extracted, ethanol precipitated reactions were run on a 7 M urea / 7% polyacrylamide gel.

b. 3' End Mapping. Two types of 3' end mapping probes were used for pACYC184 and pJJS310 RNA. The unique NcoI site within the coding sequence of the *cat* gene was used as a reference point for mapping in all cases. Full length probe was prepared by digesting pACYC184 or pJJS310 with NcoI, and the 3' recessed ends were radiolabelled. Additional probes were constructed by digesting the radiolabelled full length pACYC184 and pJJS310 probes with HindIII or AvaII, respectively. The NcoI-HindIII and NcoI-AvaII fragments were isolated by electroelution from a 0.7% agarose gel. Hybridization of RNA with the appropriate DNA probe was carried out overnight at 42°C. In hybridizations with full length probes, 1500 U of nuclease S1 were added and incubated for 2 hours at 22°C (27). Hybridization reactions in which isolated fragments served as probes were treated with 75 U of nuclease S1 for 2 hours at 37°C. The probe for 3' end analysis of pCD1018 RNA was the 1.4 kb NcoI-AvaI fragment of pCD1018 which was radiolabelled at the 3' end of the NcoI site. Hybridization of the probe and pCD1018 RNA was carried out at 45°C overnight. 20,000 U of nuclease S1 were added to the hybridization reactions and incubated for 30 min on ice (27). The phenol: chloroform (1:1) extracted, ethanol precipitated reactions were run on a 7 M urea / 7% polyacrylamide gel.

Construction of pCD1018

In order to construct a *cat* gene which contains a single transcription termination site, the 977 bp Sau3AI *cat* fragment from pACYC184 was ligated into the BamHI site of pUC119 (31) such that the 3' end of the *cat* gene was proximal to the EcoI site of the polycloning region. The resultant plasmid (pUCCAT11) was cut with SmaI and SacI, and deletion mutagenesis was carried out under conditions similar to those previously described (32). After phenol: chloroform

(1:1) extraction and ethanol precipitation, ligations to recircularize the molecules were performed. After transformation, colonies were screened for the presence of a plasmid that contained a 56 bp deletion. The resultant plasmid was pUCCAT10. Sequencing of this 3' deletion is described below.

Following EcoRI and HindIII digestion of pUC19, the 67 bp polycloning site was isolated. After addition of EcoRI linkers, the polycloning site was ligated to pUCCAT10 partially digested with EcoRI. The resultant construction was pUCCAT19. pUCCAT19 was cleaved with BamHI and filled in. The linearized plasmid was then digested with NcoI, and the 351 bp BamHI (blunt)-NcoI fragment was electroeluted from a polyacrylamide gel.

pCD10 was constructed by first filling in the unique EcoRI site of pJJS300 (33). The resultant plasmid, pCD30, was cleaved with BamHI and ligated to the 977 bp Sau3AI *cat* fragment from pACYC184. In pCD10, transcription of the *cat* gene is under control of the *lac* regulatory region. pCD10 was cleaved with SalI and filled in with Klenow. The plasmid was then cut with NcoI. The 351 bp 3' deletion fragment isolated from pUCCAT19 was then ligated to the digested pCD10. The resultant plasmid was designated pCD1019. The *rpoC* transcription terminator sequence (34), contained within a 233 bp PstI-HinfI fragment, was isolated from pAD9 (Anath Das, personal communication). The fragment was blunted by nuclease S1 and cloned into the SmaI site (within the polycloning site) of pCD1019. The resultant plasmid, pCD1018, contains a *cat* gene that has lost a portion of its 3' untranslated region and contains the *rpoC* terminator sequence. Transcription of the *cat* gene in pCD1018 is under control of the *lac* promoter/operator region, identical to the pJJS310 construction.

Decay Rate Determination

a. Dot Blot Analysis. Total RNA was extracted from exponentially growing MC1000 cells containing pACYC184, pJJS310 or pCD1018 at various times after rifampicin treatment. RNA was denatured in 6x SSC, 50% formamide, 7.4% formaldehyde and dotted onto Gene Screen Plus nylon membrane. Dilutions of an RNA sample were used as a control for linearity of probe hybridization. The 977 bp Sau3AI *cat* fragment (described above) was labelled by extension of random primers (28) and used as the probe. Conditions for hybridization were incubation overnight at 42°C in 50% formamide, 2x Denhardt, 0.5% SDS, 0.1% sodium pyrophosphate, 10% dextran sulfate and 0.2 mg/ml sonicated calf thymus DNA. As a control for quantitative RNA recovery, duplicate dot blots were probed with a 23S rRNA probe. The 1.4 kb SacI-PvuII 23S rRNA fragment was isolated from pKK3535 (35). Wash conditions were identical to those used for *cat* probed Northern hybridizations (above). Following the washes the filters were dried and counted in Ecolite scintillation cocktail. *cat* cpm were normalized to the rRNA cpm determined for the same sample. Decay rates were determined by plotting $-\ln(\text{normalized } cat \text{ cpm})$ vs time after rifampicin addition to the culture. The slope of this line is the decay constant, and the half-life of *cat* RNA is equal to $-\ln(0.5)$ divided by the decay constant.

b. Northern Analysis. Initial experiments used time points of 0, 3, 6, 10 and 15 min after rifampicin addition to the culture for pACYC184 derived RNA, while pJJS310 derived RNA was iso-

lated at 0, 2, 4, 6, and 10 min after rifampicin addition. Later experiments used 0, 0.5, 1.5, 2, 3, 5, and 6 min time points for RNA obtained from both plasmid sources. The *Sau3AI cat* fragment, labelled by extension of random primers (28), was used as a probe for Northern hybridizations. The hybridization, wash and film exposure conditions were as those described above (Detection of *cat* Specific RNA Species). The density of the resulting film bands was quantitated by densitometric scanning of the autoradiograms. Plots of $-\ln(\text{area under band})$ versus time after rifampicin addition to the culture were used to calculate the decay constants and half-lives of the messages.

c. Segmental Stability. Probe was uniformly labelled *in vivo* by growth of M13CAT-3 (see DNA Sequencing below) infected cultures of MV1190 (31) in MOPS minimal medium made 1mCi/ml with ^{32}P -orthophosphate. The radiolabelled, single stranded M13CAT-3 DNA was removed from its phage coat and digested with *HaeIII* (16). Hybridization of RNA to the probe was carried out in 0.4 M NaCl, 40 mM Pipes pH 6.4 and 1 mM EDTA at 20°C overnight. Each sample was incubated with 1200 U of nuclease S1 for 2 hours at 20°C (27). The samples were phenol: chloroform (1:1) extracted and ammonium acetate/isopropanol precipitated. Analysis was performed on an 8 M urea/6% polyacrylamide gel.

DNA Sequencing

The 977 bp *Sau3AI cat* fragment from pACYC184 was cloned into the unique *BamHI* site of M13mp10 RF. Constructions in which the non-coding strand of the fragment was in the + strand of the phage were designated M13CAT-1. Constructions in which the coding strand of the fragment was in the + strand of the phage were designated M13CAT-3. The 43 nucleotides (nt) at the 3' terminus of the *Sau3AI cat* fragment which had not been previously sequenced was determined by sequencing M13CAT-1 template (29) by the dideoxy method (36). The sequence of pACYC184 between the end of the *cat Sau3AI* fragment and the tetracycline resistance gene was also determined by the dideoxy method (36) and agreed with the recently published sequence of pACYC184 (37).

The extent of 3' terminal deletions of the *Sau3AI cat* fragment (pCD1018 construction above) were determined by dideoxy sequence analysis of single stranded DNA obtained from pUCCAT10 (31).

RESULTS

Sizing of pACYC184 and pJJS310 Derived *cat* mRNA

The *cat* specific RNA species derived from two plasmids, pACYC184 and pJJS310, were characterized. Plasmid pACYC184 is a P15A miniplasmid derivative which contains the *cat* gene from transposon Tn9 and the tetracycline resistance gene from pSC101 (24). pJJS310 is a derivative of pJJS300. pJJS300 was constructed by replacing sequences from the *EcoRI* site to the *BamHI* site in pBR322 with a 95 bp fragment containing the *lac* promoter/operator (33). pJJS310 resulted from cloning the 977 bp *Sau3AI* fragment isolated from pACYC184, which contains the complete coding sequence of *cat*, into the *BamHI* site of pJJS300 in an orientation

Figure 1 Schematic Representation of *cat* mRNA.

A. *cat* mRNA is diagrammed. The map numbers (in nt) are shown below the mRNA (as in ref. 52) while restriction sites in the corresponding DNA are shown above the mRNA; E=EcoRI, H=HaeIII, N=NcoI, P=PvuII, S1 and S2=Sau3AI. The lengths of the Sau3AI-HaeIII fragments and the internal HaeIII fragments (in nt) are also shown below the mRNA. The wavy line represents *lac* operator sequences found at the 5' end of pJJS310 derived mRNA. The 5' termini of pJJS310 *cat* mRNAs are indicated by a while the 5' terminus of *cat* mRNA derived from pACYC184 is denoted by b. The untranslated region common to all *cat* mRNAs derived from pJJS310 or pACYC184 is represented by the solid line extending from a or b, respectively, at the 5' end to sequence number 1145. In *cat* RNA derived from pACYC184, the region down-

stream of map position 1145 (S2) represents sequences from the pACYC184 plasmid. The sequences downstream of map position 1145 in pJJS310 derived *cat* mRNA represent a portion of the tetracycline resistance gene from pBR322. The coding sequence of *cat* mRNA is shown by the striped bar. The circles indicate the map positions of the 3' termini of *cat* mRNA derived from pACYC184 while the inverted triangles indicate the 3' termini of pJJS310 derived *cat* mRNA. The asterisk represents the sequence at which the *rpoC* transcription terminator was inserted in the pCD1018 construct.

B. The four transcripts derived from pJJS310 and from pACYC184 are outlined. For pJJS310, the 5' end of the transcripts is denoted by a as in Panel A, and the 3' ends of the transcripts are labelled 1a-4a (corresponds to inverted triangles in Panel A). For pACYC184, b denotes the 5' end of the messages as in Panel A, and 1b-4b identify the 3' termini (corresponds to circles in Panel A). The lengths of the transcripts as determined by nuclease S1 protection mapping are shown in nt.

C. The nucleotide sequences of the 5' untranslated region for the pJJS310 and pACYC184 derived transcripts are shown. The map numbers correspond to the map numbers in Panel A. a and b denote the 5' end of *cat* transcripts from pJJS310 and pACYC184, respectively. The sequence overscored with a dotted line is the *lacZ* ribosome binding site present on pJJS310. The sequence overscored with a solid line is the *cat* ribosome binding site. The AUG start codon for *cat* is underlined. S1 is the Sau3A1 site at the 5' end of the *cat* gene as in Panel A.

D. The nucleotide sequences of the 3' untranslated region for the *cat* transcripts from pJJS310 and pACYC184 that contain the 3' termini are presented. 1a-4a and 1b-4b represent the 3' termini mapped by nuclease S1 protection for transcripts from pJJS310 and pACYC184, respectively. Potential dyad symmetries around the termini are indicated by the arrows above the sequence. The underlined sequence is common to transcripts from both plasmids and represents the 3' end of the *cat* Sau3A1 fragment from pACYC184 (Panel A). S2 is the Sau3A1 site at the 3' end of the *cat* gene as shown in Panel A.

such that transcription of the *cat* gene is under control of the *lac* promoter (25). A restriction map of the *cat* gene from these two plasmids is shown in Figure 1. Also shown are the lengths of transcripts derived from the two *cat* genes, the nucleotide sequences of the *cat* message that are 5' to the *cat* start codon, and the nucleotide sequences of the 3' untranslated region of the *cat* messages. The *E. coli cat* gene does not contain significant secondary structure in the 5' untranslated region, and there is no evidence of translation attenuation as has been observed with the *erm* message (38) and *cat* message (17) in *B. subtilis*.

Northern blots of RNA isolated from pACYC184 and pJJS310 containing cells were probed with the radiolabelled Sau3AI *cat* fragment from pACYC184. Four major *cat* mRNA species from pACYC184 or pJJS310 were detected. Using denatured radiolabelled TaqI digested pBR322 fragments as molecular size markers, the pACYC184 *cat* species were determined to be 1225, 1150, 980 and 930 nt in length. The four pJJS310 *cat* species correspond to lengths of 1370, 1250, 1200 and 1010 nt. The results are shown in Figure 2 and summarized in Figure 1. Under the conditions of these experiments, four separate species were seen in contrast to a previous report of two *cat* species transcribed from pACYC184 (39). To verify that these species were the result of hybridization to the noncoding strand of the *cat* fragment probe, a single stranded probe was also utilized. Single stranded M13CAT-3, which contains the coding strand of the Sau3AI *cat* fragment from pACYC184 in the + strand of the phage, was used to probe identical Northern blots. Again the same four *cat* mRNA species were detected with pACYC184 and pJJS310 derived RNA while there was no hybridization with MC1000 host cell RNA (data not shown). These results indicate that each species detected represents *cat* specific mRNA.

Figure 2. Northern Hybridizations of *cat* RNA Isolated from pCD1018, pJJS310 and pACYC184 Containing Cells.

The RNA source is indicated at the top of each lane. The Sau3AI *cat* fragment isolated from pACYC184 served as the probe. The dots placed to the left of each lane indicate the *cat* mRNA species. Lane M shows the denatured TaqI digested pBR322 used as a molecular size marker. Lengths of the markers are given in nt.

5' End Mapping

End mapping analysis by nuclease S1 protection was performed to further characterize the *cat* mRNA species (40). The results of the 5' end analysis are shown in Figure 3. A 5' end-labelled 368 bp EcoRI fragment isolated from pJJS310 was used as a probe. This fragment contains the *lac* promoter/operator sequences and the initial 273 bp of the Sau3AI *cat* fragment. RNA isolated from pACYC184 or pJJS310 containing cells was hybridized to the 5' end labelled probe. A single 5' terminus of *cat* mRNAs transcribed from pACYC184 was revealed. The start point for transcription is within the Sau3AI fragment at map position 172 (see Figure 1C). The 5' end determined in this study for *in vivo* transcribed *cat* mRNA from pACYC184 was similar to (within 7 nt) that found by *in vitro* run-off transcription assays (41). Two transcriptional starts, separated by 3 nt, were detected for the *cat* transcripts from pJJS310 (Figure 3). The termini are located within the *lac* operator at map positions 129 and 132 (see Figure 1C). A third fainter band was also detected that corresponds to the length of the probe used for mapping. This fragment could be the result of incomplete probe digestion by nuclease S1 or could result from hybridization of

Figure 3 5' End Mapping of *cat* mRNA Derived from pACYC184 and pJJS310.

Lanes 2 and 3 represent 5' probe fragments protected from nuclease S1 treatment by *cat* mRNA derived from pJJS310 and pACYC184, respectively. The lengths of the fragments are shown (in nt) and correspond to the distance between the 5' termini of the mRNA and the EcoRI site within the *cat* gene. Lane 1 is a control reaction containing probe treated with nuclease S1 in the absence of RNA. Shown on the right is a M13CAT-3 sequencing reaction which served as a molecular length marker.

the probe to a low level of message that initiates near the origin of replication on the plasmid (42) and is possibly transcribed beyond the start of the *cat* gene.

3' End Mapping

The single 5' terminus detected for *cat* mRNA derived from pACYC184 and nearly identical 5' ends from pJJS310 suggested that the four *cat* species transcribed from each plasmid were the result of similar 5' ends and heterogeneous 3' ends. Initially probes for 3' end mapping experi-

Figure 4 3' End Mapping of *cat* mRNA Derived from pACYC184 and pJJS310.

Lanes 1 and 4 represent 3' probe fragments protected from nuclease S1 treatment by pACYC184 or pJJS310 derived *cat* mRNA, respectively. The lengths of these fragments (in nt) are shown and correspond to the distance between the NcoI site within the *cat* gene to the 3' ends of the mRNA. Lanes 2 and 3 represent control reactions containing the pACYC184 or pJJS310 3' RNA probes, respectively, treated with nuclease S1 in the absence of corresponding RNA. A sequencing reaction of the M13CAT-3 served as a molecular length marker.

ments were full length linearized pACYC184 and pJJS310 plasmid DNA radiolabelled at the NcoI site (see Figure 1). As seen in Figure 4, four major termini were detected by nuclease S1 protection mapping for *cat* RNA derived from cells containing pACYC184. Four major DNA fragments were also protected from nuclease S1 by *cat* mRNA isolated from pJJS310 containing cells. In both cases minor bands were detected but were not characterized. In support of these results, either a 2.4 kb NcoI- HindIII fragment isolated from pACYC184 or a 1.4 kb NcoI-AvaI DNA fragment isolated from pJJS310 were utilized as probes. Each fragment was labelled at the

Table 1. Half-Lives of *cat* mRNA Species Determined by Northern and Dot Hybridization.

mRNA source	<i>cat</i> mRNA Species Lengths (nt) as Determined by: ^a		Half-Life (min) as Determined by: ^b	
	Northern	S1 mapping	Dots	Northern
pACYC184	930	940	1.8	2.4 ± 1.0
	980	989		2.0 ± 0.4
	1150	1159		1.0 ± 0.6
	1225	1253		1.4 ± 0.4
pJJS310	1010	983	2.2	1.5 ± 0.5
	1200	1182		1.4 ± 0.6
	1250	1232		1.9 ± 1.4
	1370	1322		1.2 ± 0.4
pCD1018	1100	1118	2.4	1.7 ± 0.7

a *cat* mRNA species lengths determined by Northern hybridizations are the average of at least three independent measurements. Lengths were also calculated from nuclease S1 analyses of 5' and 3' ends.

b Half-lives measured by dot hybridization are the average of duplicate blots. The half-life measurements and standard deviations for Northern analysis are the result of at least three separate determinations.

NcoI site and hybridized with the appropriate RNA. Identical results were obtained (data not shown).

Full lengths of the mRNA species were calculated using the measured 3' end fragment lengths and the previously determined major 5' termini. The calculated lengths of these four longest transcripts compared very closely with the lengths of the four predominant *cat* mRNA species determined by Northern hybridization as shown in Table 1. The actual lengths of the transcripts from pACYC184 are 940, 989, 1159 and 1253 nt as calculated by nuclease S1 mapping. The transcripts from pJJS310 were calculated to be 983, 1182, 1232, 1322 nt in length. The 940 nt transcript from pACYC184 and the 983 nt transcript from pJJS310 contain identical 3' termini. The common 374 nt 3' protected fragment maps within the 3' untranslated portion of the transcript and corresponds to map position 1112 of the Sau3AI *cat* fragment (Figure 1A). The end of this transcript maps to the 3' side of a stem-loop structure with a ΔG value of -11 kcal (Figure 1D). The difference in length of these species can be accounted for by the 43 nucleotides of the *lac* operator sequence and the *cat* mRNA untranslated sequence transcribed at the 5' end of messages from the pJJS310 plasmid. These 43 nt are not present in the pACYC184 derived *cat* transcripts (Figure 1C).

The remaining six *cat* mRNA species derived from pACYC184 and pJJS310 have divergent 3' termini. These major termini map outside the Sau3AI *cat* fragment in the corresponding vector sequences. The 3' termini of the three longest *cat* transcripts from pACYC184 lie in the region

Figure 5 Northern Hybridizations Demonstrating Decay of *cat* mRNA Derived from pACYC184 and pJJS310.

A. Northern blot of pACYC184 derived *cat* mRNA isolated at various times after rifampicin addition to the culture (time in min indicated at the top of the figure). The *cat* specific species and their molecular lengths in nt are indicated.

B. Decay of pJJS310 RNA as seen by Northern blot. The figure description is as in A.

downstream of the *cat* gene at sequence number 1161, 1331, and 1425 (refer to Figure 1A).

Computer analysis showed that the termini corresponding to the two longest transcripts were within regions which have potential secondary structure (ΔG values of -23 and -14 kcal, respectively). No significant secondary structure was found around the 3' terminus of the 989 nt transcript (Figure 1D). The 3' termini of the three longest pJJS310 derived *cat* RNAs lie within the tetracycline resistance gene of pBR322 (43). These termini map to sequence numbers 1311, 1361 and 1451 as shown in Figure 1A. The 3' end sequences of pJJS310 *cat* mRNA at map positions 1311 and 1361 terminate within regions of pBR322 which have potential secondary structures with ΔG values of -16 and -11 kcal, respectively. The 3' terminus at sequence number 1451 does not correspond to a region of apparent secondary structure (Figure 1D).

Decay Rate Determination of *cat* mRNA from pACYC184 and pJJS310

Degradation of *cat* mRNA was analyzed by two methods. Dot blot analysis provides a means of measuring the rate at which mass of *cat* mRNA is lost while Northern hybridizations measure the rate at which an individual full length mRNA species is lost. A summary of the decay analyses is presented in Table 1. Half-lives of the four distinct pACYC184 *cat* species as determined by Northern hybridization (Figure 5A) reveal no significant differences in decay between the species. Similarly, there appeared to be no significant difference between the half-lives of the four pJJS310 *cat* mRNA species (Figure 5B). The four species from each plasmid contain similar 5' termini but have divergent 3' ends. By examining the decay rates of *cat* mRNA species derived from a single plasmid it appears that divergent 3' ends do not significantly affect the degra-

dation of the message. In addition, the decay rate of the shortest species derived from each plasmid (940 nt from pACYC184 and 983 nt from pJJS310) does not appear to differ significantly. Except for the additional 43 nt at its 5' terminus, the 983 nt species from pJJS310 is identical to the 940 nt species from pACYC184. As with the 3' termini, it appears that these sequences at the 5' end do not affect the degradation rate of *cat* mRNA. The results from the Northern analysis suggest that the length or composition of the 5' or 3' untranslated region has no effect on degradation of *cat* mRNA. In addition, there was no significant difference in mass decay values for pACYC184 and pJJS310 derived *cat* mRNAs.

Single *cat* RNA Species Transcribed from pCD1018

The mapping experiments done thus far have used mRNA transcribed from pACYC184 and pJJS310 plasmids, each of which specify at least three *cat* mRNA species which terminate outside of the Sau3AI fragment. In order to simplify analysis and further investigate the effects of the 3' terminus on message decay, a plasmid was constructed which would generate a single *cat* RNA transcript. The construction involved modification of the 3' terminus of the *cat* message with the addition of the rho independent transcription termination sequence from the *rpoC* gene (34). This sequence contains three regions capable of forming stable stem and loop structures. The construction of the pCD1018 plasmid is described in Materials and Methods.

As shown in Figure 2 there is one major *cat* mRNA species transcribed from pCD1018 that is 1100 nt in length. Nuclease S1 analysis was used to verify the location of the 3' end of pCD1018 *cat* mRNA. A 3' end labelled 1.4 kb NcoI-AvaII fragment isolated from pCD1018 was used as the probe. Figure 6 shows the results of this analysis. The major protected DNA fragment detected was 508 nt in length and represents a single 3' terminus generated within the *rpoC* terminator. The length of pCD1018 *cat* mRNA calculated from the S1 analysis is 1118 nt and is in good agreement with the length measured by Northern hybridization.

Decay rates of the *cat* RNA derived from pCD1018 were determined by dot and Northern hybridizations (Table 1). Alteration of the 3' terminus by addition of the *rpoC* termination sequence does not affect the decay of the *cat* mRNA significantly when compared to the decay of the *cat* mRNAs transcribed from the pACYC184 and pJJS310 plasmids. The insertion of this stable stem and loop structure at the 3' end of an mRNA species does not appear to provide stabilization of the upstream sequences. This result suggests that the rate limiting degradation events which occur within *cat* mRNA are independent of this structure, perhaps due to the involvement of sequences upstream of the terminator region.

Segmental Stability of *cat* mRNA

The stability of individual domains of the *cat* message was measured (16). These measurements can detect a segment of the message that is more stable than the rest of the mRNA thereby localizing a potential barrier to decay. Single stranded M13CAT-3 (see Materials and Methods) was uniformly radiolabelled and cleaved with HaeIII. This mixture of HaeIII fragments was hybridized in five-fold molar excess to RNA samples which were extracted from cells containing pACYC184 at various times after the addition of rifampicin. Following nuclease S1 treatment,

Figure 6 3' End Mapping of *cat* mRNA Derived from pCD1018.

Lane 1 represents a 3' probe fragment protected from nuclease S1 treatment by pCD1018 derived *cat* RNA. The length of the fragment (in nt) is shown and corresponds to the distance between the NcoI site within the *cat* gene to the 3' end of the mRNA. Lane 2 represents a control reaction in which the pCD1018 3' RNA probe, in the absence of corresponding RNA, was treated with nuclease S1. A sequencing reaction of the M13CAT-3 served as a molecular length marker.

the hybrids were analyzed on denaturing polyacrylamide gels (Figure 7). More accurate length measurements were obtained by running the samples on a denaturing polyacrylamide gel next to a sequencing ladder (data not shown). *cat* transcripts from pACYC184 protected HaeIII fragments 45, 87, 178, 222, 392, and 441 nt in length. Figure 1A diagrams the position of the HaeIII protected fragments. The 178 nt fragment represents sequences from the 5' end of the transcript to the first HaeIII site. Internal sequences correspond to HaeIII fragments of 45, 222, and 87 nt in length. Fragments which are 392 nt and 441 nt in length represent protection by the 3' end regions of the message. The 392 nt segment is composed of sequences from the fourth to the fifth HaeIII sites at map positions 704 and 1096. The 441 nt fragment likely overlaps with the 392 nt fragment. This protected fragment includes sequences from the HaeIII site at map position 704 to the Sau3AI site at map position 1145. This DNA probe extending from sequence number 704 located within the *cat* gene to a HaeIII site within the M13 vector was probably a product of partial HaeIII digestion. A band 172 nt in length was also detected but its origin not determined.

The sum of the intensities of the 441 and 392 nt fragments were less than the intensity of individual HaeIII fragments of shorter length. To ensure that these probe fragments were in excess

Figure 7 Nuclease S1 Analysis Demonstrating Decay of Various Segments of *cat* mRNA Derived from pACYC184.

cat mRNA derived from pACYC184 was isolated from cells at various times after rifampicin addition to the culture (time in min indicated at top of figure). The lengths (in nt) of the HaeIII probes (see text) protected from nuclease S1 treatment by *cat* RNA are indicated. The last lane (C) represents a control reaction in which the probe, in the absence of pACYC184 RNA, was treated with nuclease S1.

in the hybridization conditions, similar experiments were run with twice the amount of HaeIII digested M13CAT-3 (ten-fold molar excess), and similar results were obtained (data not shown). The discrepancy in intensities of the 441 and 392 nt fragments was also seen in the HaeIII digested probe alone and may reflect the inefficiency of enzymatic cleavage at the *cat* HaeIII site (sequence number 1096) and at the M13 HaeIII site 3' to the *cat* fragment.

The half-lives of the six *cat* RNA domains do not vary significantly from each other (Table 2). In this manner, decay of the *cat* mRNA appears to be similar to the decay mode of the *bla* transcript (16). As in the case of *bla* RNA, however, the inaccuracies of segmental decay rate measurements may obscure the detection of small differences in the decay rates between message domains. The uniformity of decay found throughout the message indicates that there are no major barriers to decay within the message. This suggests that once an initial degradation event occurs, the different domains of the message decay at similar rates.

Table 2 Segmental Decay Rates of pACYC184 *cat* mRNA.

Protected HaeIII Fragment Length (nt)	Half-Life (min)
441	1.3 $\pm$ 0.1
392	1.6 $\pm$ 0.5
222	1.4 $\pm$ 0.1
178	0.9 $\pm$ 0.1
87	1.7 $\pm$ 0.6
45	1.1

The lengths of the HaeIII protected probe fragments and the half-lives of the corresponding regions of mRNA are indicated. Half-life measurements are the average of two determinations except for the 45 nt fragment, and the standard deviations are indicated.

DISCUSSION

There does not appear to be a single region or sequence common to all mRNAs which regulates decay. Control of prokaryotic monocistronic mRNA degradation can reside in sequences located at the 5' terminus (15) and 3' terminus (7) of the message. Relative expression and decay of portions of polycistronic mRNAs may be controlled by intercistronic cleavage (14,18-21,44,45). The manner in which RNA is degraded is not completely defined even though models have been proposed for this process (2,20,22,46). Endonucleolytic cleavages by RNaseIII or uncharacterized RNases can affect the decay of mRNA (13,15). In *E. coli*, polynucleotide phosphorylase and RNaseII are also involved in degradation of mRNA (47,48). These enzymes have a 3' to 5' exonuclease activity. No 5' to 3' exonuclease activity has been identified in *E. coli*. Because exonucleases are involved in degradation of *E. coli* mRNA, the termini of an mRNA molecule may play an important role in establishing the overall decay rate. The *cat* gene, located in plasmids pACYC184, pJS310 and pCD1018, produces *cat* mRNAs which vary in length and composition at their 5' and 3' termini. These constructs provided a means of investigating the effect of terminal sequences on the decay of this message.

Analysis of *cat* mRNAs transcribed from pACYC184, pJS310 or pCD1018 indicates that the 3' terminal nucleotide sequence does not significantly affect the decay rate of the *cat* message. It might be expected that the addition of up to several hundred nucleotides to the 3' end of the message would affect the stability if degradation involved a number of random cleavages in which all nucleotidyl bonds were susceptible. However, this does not appear to be the case. The length of a *cat* mRNA species does not appear to affect its decay rate in contrast to the proposed length dependent decay rate of yeast mRNA (6). It might also be expected that if decay begins at the 3' terminus, addition of a large stem and loop structure would present a barrier to 3' to 5' exonucleolytic attack. The presence of a rho independent terminator has been reported to contribute to the stability of an mRNA species (49,50), and other stem and loop structures have been observed to stabilize upstream sequences (7). The addition of the *rpoC* rho independent transcription ter-

minator to the 3' end of *cat* mRNA in pCD1018 does not significantly affect the stability of upstream sequences. Failure of the *rpoC* termination stem and loop structure of pCD1018 to cause some alteration in the decay rate may be attributed to the ineffectiveness of this particular structure to act as a message stabilizer (9), particularly if the rate-limiting decay event does not involve sequences at the 3' end of the message. Similar arguments can be made for the secondary structures found at some of the 3' termini of *cat* messages transcribed from pJJS310 and pACYC184. Even though this analysis does not exclude the possibility that the 3' end sequence is important to determining the decay rate, it does suggest that the rate determining decay event(s) occurs in sequences other than those at the end of the message.

The *cat* mRNA species derived from pJJS310 have an additional 43 nt at the 5' terminus compared to the *cat* mRNA species derived from pACYC184. One RNA species transcribed from each plasmid shares a common 3' end. The similar decay rates of these species, which share a common 3' terminus, suggest that the additional sequences in the 5' untranslated region do not affect the turnover rate of the message. It is possible that the *lac* operator sequence contains some degradative control region. The *lac* operator sequence can form a stem and loop structure, and mutations in this region have been shown to alter the half-life of the *lac* message (5). If the *cat* mRNA decay is influenced by events occurring in this region it would probably be difficult to detect. The half-life of the *lac Z* message is approximately 1.5 minutes (20), within the same range as the half-life of the *cat* message. In the *lac* operator-*cat* fusion there may be separate "initial" decay events occurring (one in the operator sequence, one in *cat*), but because of the similar decay rates of the *lac* and the *cat* messages, the overall decay rate is the same as in *cat* mRNA alone. However, it is apparent that added *lac* operator sequences do not interact with *cat* mRNA sequences to form new decay determinants which confer a significantly different turnover rate to the chimeric message.

In addition to studying the rate of decay of full length message, the stabilities of different domains of the *cat* message (16) were measured. Once processive exonucleolytic digestion of the message is initiated, then the decay rate of various domains of the message should be approximately the same as long as no barriers to exonuclease activity are encountered within the message. If stabilities of different domains of the message are not equivalent, then the presence of a barrier to exonuclease digestion may be indicated. These types of barriers have been reported and include the formation of secondary structures (8,51). Analysis of the various *cat* mRNA segmental decay rates revealed no significant differences in half-life between regions of the message. These results are consistent with a decay process that involves an initial rate-limiting event. Once this initial event occurs, all domains of the *cat* message are equally susceptible to decay. These experiments do not directly identify the nature of the rate-limiting event. However, *cat* messages with variable lengths and composition of 5' and 3' untranslated sequence having similar decay rates suggests that the rate-limiting event occurs within a region of the message that is common to all of the transcripts. This rate-limiting event would most likely involve an endonucleolytic cleavage internal to the message followed by exonuclease digestion of the cleavage

products. Our conclusions are consistent with the model for mRNA decay proposed for the *lac* message (2,22). Within the *lac* message, endonucleolytic cleavages have been defined by sequencing the ends of the cleavage products. While there is thought to be no one unique site that represents the first cleavage, any one of several cleavages would effectively inactivate the message and initiate mass decay.

The experiments in this study measured the chemical stabilities of full length mRNA species; they did not address the functionality of the message. Functionality, also measured in terms of half-life, describes the ability of a message to be maintained as a template for translation. The functional half-life of *cat* mRNA from pACYC184 containing cells in exponential growth has been determined to be about 0.7 min (Kuzj and Schottel, manuscript submitted). The functional decay rate of the *cat* mRNA is approximately the same as the rate of loss of full length species detected by Northern hybridization. The loss of functionality therefore appears to be correlated with an event which also results in loss of the intact mRNA. If this functional inactivation involves either 3' to 5' exonucleolytic activity or an endonucleolytic cleavage within the 3' end of the coding sequence, we might expect to see CAT protein species smaller than full length by immunoprecipitation. There is no evidence for the production of truncated polypeptides (A. Kuzj, personal communication). However, an endonucleolytic cleavage near the 5' end of the message would simultaneously inactivate the message for translation and would result in loss of full length message detectable by Northern hybridization. A cleavage event near the ribosome binding site of the message could represent the initial event in decay, followed quickly by mass degradation events which include both endonucleolytic and exonucleolytic cleavages.

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