Homeotic gene *Antennapedia* mRNA contains 5'-noncoding sequences that confer translational initiation by internal ribosome binding

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The *Antennapedia* (*Antp*) homeotic gene of *Drosophila melanogaster* has two promoters, P1 and P2. The resulting *Antp* mRNAs contain 1512-nucleotide (P1) and 1727-nucleotide (P2) 5'-noncoding regions, composed of exons A, B, D, and E (P1) or exons C, D, and E (P2), respectively. Multiple AUG codons are present in exons A, B, and C. We have found that 252-nucleotide exon D, common to mRNAs from both transcription units and devoid of AUG codons, can mediate initiation of translation by internal ribosome binding in cultured cells. Many mRNAs in *Drosophila* contain long 5'-noncoding regions with apparently unused AUG codons, suggesting that internal ribosome binding may be a common mechanism of translational initiation, and possibly its regulation, in *Drosophila*.

[**Key Words:** Internal ribosome binding; homeotic gene; *Antennapedia* mRNA; dicistronic mRNA; RNA transfection]

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The homeotic genes required for segments in *Drosophila melanogaster* to have different developmental fates are expressed in some segment primordia but not in others. Each segmented part of the embryo expresses a different homeotic gene, or combination of them (for review, see Duncan 1987; Mahaffey and Kaufman 1988). Transcription of the homeotic genes is tightly regulated by the activities of a large number of regulators, including many of the segmentation genes (Scott and Carroll 1987; Ingham 1988; Irish et al. 1989). Transcription of a homeotic gene in a location where the gene is normally silent can lead to transformations of segments (Frischer et al. 1986; Schneuwly et al. 1987a,b). Thus, transcriptional regulation is clearly involved in the spatially regulated activities of homeotic genes. The sequence organization of certain homeotic mRNAs suggests that there may be an additional level of regulation.

Transcription of the homeotic *Antennapedia* (*Antp*) gene is initiated at two promoters (P1 and P2), producing transcripts that differ in their 5'-noncoding regions (5' NCR) (Laughon et al. 1986; Schneuwly et al. 1986; Strocher et al. 1986). Figure 1 illustrates that P1-initiated transcripts contain a 1512-nucleotide 5' NCR derived from sequences in non-protein-coding exons A, B, D, and E, whereas P2-initiated transcripts contain a 1727-nucleotide 5' NCR derived from exons C, D, and E. The non-coding exons A and B contain 8 AUG codons preceding the translation initiator AUG codon located in exon E;

similarly, exon C contains 15 upstream AUG codons. All of these upstream AUG codons are followed by very short open reading frames that could potentially encode peptides ranging from 6 to 44 amino acids. Noncoding exon D and the 5' half of exon E, common to mRNAs initiated at both P1 and P2, are devoid of AUG codons (Laughon et al. 1986; Strocher et al. 1986). It is remarkable that the large number of total upstream AUG codons in these 5'-noncoding regions does not prevent translation of the main open reading frame. According to the scanning model for translational initiation (Kozak 1989, 1991), translational initiation of *Antp* mRNA should be very inefficient.

If the scanning mechanism were used for translational initiation of *Antp* mRNA, derived from the P2 promoter, for example, the ribosomal 43S ternary complex would bind at the 5' end of the mRNA and would scan 1730 nucleotides of the 5' NCR, bypassing 15 AUG codons, to initiate protein synthesis at the sixteenth AUG codon. Several of the 15 upstream AUG codons are in a favorable context to initiate protein synthesis in *Drosophila* cells (Table 3, below; Cavener 1987). In addition, RNA structures in this long leader may render the scanning of the ribosomal subunits inefficient.

There are precedents for translational initiation in mRNAs with multiple AUG codons in their 5' NCRs, by both ribosomal reinitiation and internal ribosome binding mechanisms. For example, yeast GCN4 mRNA con-

Figure 1. Coding and noncoding exons of *Antp* mRNAs. Structure of *Antp* transcripts derived from the P1 and P2 promoters. (Open rectangles) Noncoding exons; (solid rectangles) coding exons. Exons are numbered according to Laughon et al. (1986).

tains 4 AUG codons upstream of the initiator AUG codon, and this property is responsible for the regulation of GCN4 translation initiation in response to amino acid availability (for review, see Hinnebusch 1988). Translational initiation by internal ribosome binding was first observed in two picornaviral mRNAs. Pelletier and Sonenberg (1988) and Jang et al. (1989) found that the 5' NCRs of poliovirus and encephalomyocarditis virus (EMCV), respectively, could mediate translational initiation by internal ribosome binding. Because internal ribosome binding to viral RNAs was found to function both in uninfected and infected cells, it was concluded that the mammalian cells possess all of the factors required for this mechanism. Recently, the 5' NCR of a cellular mRNA was found to direct translation by internal ribosome binding in human cells (Macejak and Sarnow 1991).

We tested whether ribosomes could use internal sequences within the 5' NCR of *Antp* P2 mRNA to initiate translation in dicistronic transcripts. We found an apparent internal ribosome entry site (IRES) sequence element within exon D that can direct translation initiation at the most proximal downstream AUG codon in cultured *Drosophila* cells. That at least 20% of *Drosophila* mRNAs contain long leaders with multiple upstream AUG codons suggests that internal ribosome binding may be an important mechanism in the expression of regulatory genes in the organism.

Results

The 5' NCR of Antp P2 mRNA mediates translation of the second cistron in dicistronic mRNAs in cultured Drosophila cells

The 5' NCR of *Antp* mRNAs revealed a sequence organization similar to that of the 5' NCR of poliovirus; both 5' NCRs are unusually long [1730 (P2) and 743, respectively] and contain multiple upstream AUG codons [15 (P2) and 8, respectively]. To test whether sequences in the 5' NCR of *Antp* enable translation to be initiated by internal ribosome binding, we constructed plasmid vectors (Fig. 2) similar to the ones first described by Pelletier

and Sonenberg (1988). Briefly, polymerase II in cells transfected with these plasmids should initiate transcription at the SV40 promoter, producing a capped dicistronic transcript that contains 3'-terminal polyadenosine. The first cistron, encoding chloramphenicol acetyltransferase (CAT), lies downstream of a short, capped 5' NCR and should be translated by the conventional cap-dependent scanning mechanism (Kozak 1989). However, the second cistron, encoding luciferase (LUC), should be translated only if the preceding intercistronic spacer (ICS) insert contains an IRES, a sequence that can mediate internal ribosome binding.

We engineered different parts of the 5' NCR of *Antp* P2 mRNA, as well as control sequences, into the ICS region of pSV_ACAT/ICS/LUC (Fig. 3) and transfected the individual plasmids into *Drosophila* Schneider line 2 (SL2) cells. The translation products of the first (CAT) and the second (LUC) cistrons were monitored during the transient expression of the transfected plasmids. As expected, all dicistronic mRNAs directed the synthesis of similar amounts of active CAT protein in transfected cells (Table 1). Therefore, the different amounts of CAT protein that accumulated in individual transfection experiments can be used as an internal control for the different transfection efficiencies of the plasmids and for the amount of translation-competent mRNA. If these mRNAs are intact, relative CAT activity can serve as a control for RNA stability as well.

The accumulation of LUC activity in the same lysates was then determined (Table 1). Dicistronic mRNAs containing the entire 5' NCR of *Antp* P2 (AP2, exons C, D, and E) in the ICS directed the translation of the LUC protein at a 240-fold level higher than dicistronic mRNAs containing control sequences in the ICS (exons D–E inverted, $\overline{DE}$; Table 1). Monocistronic mRNAs containing the 5' NCR of *Antp* upstream of LUC produced the same amount of active LUC (not shown), indicating that translation of both monocistronic and dicistronic mRNAs was initiated by the same major route. When the activity of translation products from dicistronic mRNAs bearing only exons D and E in the ICS was monitored, LUC activity was found to be 30% the level resulting from mRNAs containing exons C, D, and E in the ICS (Table 1). Thus, RNA sequences in exons D and E are sufficient to direct translation of the second cistron in dicistronic mRNAs in SL2 cells.

Direct transfection of dicistronic mRNAs into cultured cells: exon D of Antp is sufficient for translation of the second cistron

To substantiate these data further and to exclude the possibility that cryptic promoter sequences in the 5' NCR of the *Antp* cDNA produced functionally monocistronic LUC mRNA, we transfected dicistronic mRNAs, synthesized in vitro, directly into SL2 cells. Capped dicistronic mRNAs containing 3'-terminal polyadenosine tails were synthesized in vitro by use of T7 RNA polymerase and transfected directly into SL2 cells. All mRNAs tested were functionally associated with ribo-

Figure 2. Structure of DNA expression vectors used to produce dicistronic mRNA molecules. Structure of a generic vector to express dicistronic mRNAs in cultured cells. The plasmid, pSV_ACAT/ICS/LUC, contains promoter and enhancer elements from SV40, followed by the coding region for CAT, an ICS, and a second cistron encoding LUC. [ivs and poly(A)] Cassettes containing sequences mediating splicing and polyadenylation, respectively, of the primary transcript. The predicted structure of the primary transcript is shown. (Cap) The m⁷GpppN structure (N can be any nucleotide) found at the 5' end of polymerase II transcripts.

somes and were translated to produce CAT, the product of the first cistron (Table 2). The amount of CAT activity in cell lysates varied slightly between different RNA species tested. Most likely, differences in the sequences and structures of the RNA species resulted in different effi-

ciencies of RNA uptake into the cells or in different stabilities of the RNAs in the cells. In any case, determination of LUC activity in the same lysates revealed much greater differences. RNAs containing exons C, D, and E (AP2), exons D and E (DE), or exon D alone (D) in the ICS

Figure 3. Structures of the ICS regions in dicistronic mRNAs. Dicistronic mRNAs containing different parts of the *Antp* P2 5' NCR are shown. The 3' and 5' ends of the CAT- and LUC-coding regions, respectively, are indicated. *Antp* P2 contains the entire 5' NCR of *Antp* P2 (exons C, D, and E); exons $\overleftarrow{D-E}$ contain exons D and E inverted; exons $\overleftarrow{D-E} + \text{AUG}$ contain an AUG triplet in the same translational reading frame as the AUG codon used to translate LUC. (↓) AUG codons.

Table 1. Translation of dicistronic mRNAs after transfection of DNA plasmids into cultured *D. melanogaster* SL2 cells

Plasmid	Chloramphenicol conversion/ 10^6 cells (%)	LUC light units/ 10^6 cells
AP2 ^a	10 $\pm$ 2	220,000 $\pm$ 20,000
$\overline{DE}$ ^b	12 $\pm$ 2	76,500 $\pm$ 6,500
$\overline{DE}$ ^a	9 $\pm$ 2	900 $\pm$ 300
$\overline{DE}$ + AUG ^a	12 $\pm$ 3	190 $\pm$ 10

Structural features of plasmids are shown in Fig. 3. Experiments were repeated three (a) or six (b) times.

region, mediated high levels of translation of the second LUC cistron (Table 2). Also, uncapped AP2 RNAs mediated high levels of translation of LUC, but not of CAT, suggesting that the second cistron can be translated independently of the first cistron. However, exon E (E), exon C (C), inverted exons D and E ($\overline{DE}$), or DE sequences containing an upstream AUG codon (see Fig. 3) did not direct translation of LUC when present in the ICS region. The entire 5' NCR of *Antp P2* (exons C, D, and E) again mediated translation of the second LUC cistron most efficiently, but exon D alone was clearly sufficient to mediate translation of LUC. Thus, these results exclude the possibility that cryptic promoter elements present in the first cistron or in the ICS region in the DNA transfection experiments could have produced functionally monocistronic mRNAs.

There are three possible mechanisms by which the 252-nucleotide RNA sequence (exon D) of the 5' NCR of *Antp P2*, when present as an ICS, could stimulate translation of the second cistron. First, a nuclease-sensitive site in the ICS could allow the production of functionally monocistronic mRNAs. Second, the 5' NCR could provide RNA structures or sequences that mediate readthrough of ribosomes from the first (CAT) to the second LUC cistron. Alternatively, the *Antp* sequences could provide an IRES element for the LUC cistron (Pelletier and Sonenberg 1988; Jang et al. 1989).

Intact dicistronic 5' CAT/exon DE/LUC 3' mRNA is present in transfected SL2 cells

We analyzed the integrity of dicistronic mRNAs expressed from pSV_ACAT/exon DE/LUC-transfected SL2 cells. Briefly, SL2 cells were transfected with pSV_ACAT/exons DE/LUC containing an inverted repeat upstream of CAT, which is predicted to result in the formation of an RNA hairpin structure in the 5' NCR of CAT, as discussed in the next section. At 48 hr after transfection, polyadenylate-containing RNA was prepared and was examined by a ribonuclease protection assay (Zinn et al. 1983). A 711-nucleotide radiolabeled RNA probe (Fig. 4A) was hybridized to oligo(dT)-selected mRNA obtained from transfected lysates, and an ~635-nucleotide-long RNA was protected from nuclease degradation (Fig. 4B, lane 4), demonstrating that the dicistronic mRNA

was intact. Furthermore, no protection was observed when mRNAs from nontransfected cells were used in the protection assay (Fig. 4B, lane 3), excluding the possibility that protection from fortuitously cross-hybridizing cellular mRNA was monitored. Similarly, transfection of pSV_ACAT/exons DE/LUC into mammalian COS cells resulted in the production of intact dicistronic mRNAs (Fig. 4C, lane 3) which could be translated to produce LUC (not shown).

Furthermore, we transfected gel-purified, dicistronic 5' CAT/exon DE/LUC 3' mRNAs directly into SL2 cells and analyzed the integrity of the dicistronic RNAs at 19 hr after transfection. Figure 4C (lane 5) shows that a 635-nucleotide fragment could be protected from ribonuclease digestion, indicating that dicistronic mRNAs containing intact intercistronic spacer regions were present at that time after transfection.

Translation of the second cistron in dicistronic mRNAs is due to internal ribosome binding

To determine whether the second cistron in the dicistronic mRNA is translated by ribosomal readthrough or by internal ribosome binding, we constructed a dicistronic vector containing exon DE sequences in the ICS but also bearing an inverted repeat DNA element upstream of the CAT-coding region. After transfection of this DNA into cultured cells, the transcript produced should contain a stable RNA hairpin in the 5' NCR of CAT. Because stable RNA hairpins in 5' NCRs are known to inhibit translation, the CAT cistron of this mRNA should be poorly translated, as we have shown previously (Macejak and Sarnow 1991).

We reasoned that if exon DE sequences mediate translation of LUC by internal ribosome binding, an RNA hairpin structure upstream of CAT, to inhibit the translation of CAT, should not affect the translation of LUC. In contrast, if translation of LUC in dicistronic constructs is accomplished by ribosomal readthrough, sec-

Table 2. Translation of dicistronic mRNAs after direct transfection of RNA molecules into cultured *D. melanogaster* SL2 cells

RNA	Chloramphenicol conversion/ 10^6 cells (%)	LUC light units/ 10^6 cells
AP2	68 $\pm$ 2	16,000 $\pm$ 2,000
$\overline{DE}$	67 $\pm$ 3	7,000 $\pm$ 1,000
D	49 $\pm$ 5	3,500 $\pm$ 500
E	49 $\pm$ 2	240 $\pm$ 200
C	30 $\pm$ 2	300 $\pm$ 100
$\overline{DE}$	60 $\pm$ 3	240 $\pm$ 40
$\overline{DE}$ + AUG	66 $\pm$ 3	170 $\pm$ 60
Uncapped AP2	9	13,600

All dicistronic mRNAs contained a m⁷GpppG cap structure, except uncapped AP2. Structural features of dicistronic mRNAs are shown in Fig. 3. Experiments were repeated three times, except the transfection with uncapped AP2 (1 $\times$).

Figure 4. Ribonuclease protection analysis of dicistronic CAT/exon DE/LUC mRNA containing an RNA hairpin upstream of the CAT-coding region. (A) Diagram of dicistronic CAT/exon DE/LUC mRNA hybridized to antisense RNA probe. Uniformly ^{32}P -labeled RNA probe (771 nucleotides), complementary to 635 nucleotides of the dicistronic mRNA and corresponding to the plasmid DNA between the *Nco*I site (N) in CAT and the *Xba*I site (X) in LUC, was synthesized *in vitro*. The ICS region containing exons D and E is 450 nucleotides in length. (B) Ribonuclease protection analysis of dicistronic mRNAs obtained from SL2 extracts, transfected with pSV_A hairpin CAT/exon DE/LUC plasmids. Polyadenylate-containing mRNAs were isolated from transfected SL2 cell extracts and treated as indicated in Materials and methods. The reaction products were analyzed in denaturing polyacrylamide gels. A composite autoradiograph of the gel is shown. Lanes 1–3 were exposed for 14 hr; lane 4, for 4 days. Note that the gel displaying lanes 1–3 was left in developing solution longer than the gel showing lane 4. (Lane 1) RNA probe alone; (lane 2) RNA probe after ribonuclease treatment; (lane 3) RNA probe hybridized to total RNA isolated from mock-transfected SL2 cells; (lane 4) RNA probe hybridized to poly(A)-containing RNA isolated from cell extracts, transfected with pSV_ACAT/exon DE/LUC containing an RNA hairpin upstream of the CAT-coding region. The migration of *in vitro*-synthesized, labeled RNA molecules of known length (in nucleotides) is shown at *left*. (C) Ribonuclease protection analysis of dicistronic mRNAs obtained from COS or SL2 cell extracts. COS cells were transfected with pSV_A hairpin CAT/exon DE/LUC plasmids; alternatively, *in vitro*-synthesized, gel-purified dicistronic mRNAs were transfected into SL2 cells. Cell extracts were prepared and RNAs were analyzed by ribonuclease protection assays, as described for B. A composite autoradiograph (lanes 1–4 were exposed for 14 hr; lanes 5–7 for 30 hr) of the gel is shown. (Lane 1) RNA probe alone; (lane 2) RNA probe after nuclease treatment; (lane 3) RNA probe hybridized to mRNA isolated from transfected COS extracts; (lane 4) no sample; (lane 5) RNA probe hybridized to RNA isolated from transfected SL2 extracts; (lanes 6,7) *in vitro*-

synthesized, labeled RNA molecules of known length. The sizes (nucleotides) of the marker RNAs are indicated at *left*. The RNA species in lane 5, migrating below the 600-nucleotide marker RNA, resulted from ribonuclease cleavage in an AU-rich region in the CAT gene [Macejak and Sarnow 1991]. This RNA species can also be seen in lane 3 after longer exposure of this part of the gel.

and cistron translation should be inhibited by the RNA hairpin at the 5' end of the first cistron. Thus, we tested whether dicistronic 5' (RNA hairpin) CAT/exon DE/LUC 3' mRNAs are associated with polysomes under conditions where no ribosome binding would occur at the 5' ends of the mRNAs. Such a condition can be mimicked by infection of cells with poliovirus. Infection of mammalian cells with poliovirus is known to result in the dramatic inhibition of host cell protein synthesis (for review, see Sonenberg 1987). This inhibition is the result of the proteolytic cleavage of the p220 component of the cap-binding protein complex eIF-4F [Etchison et al. 1982]. As a consequence, it is thought that the 43S ternary complex cannot be recruited to the 5' end of capped cellular mRNAs, and cellular mRNAs are not associated with polysomes in poliovirus-infected cells. However, poliovirus mRNA, translated by internal ribosome binding, is found to be associated with polysomes when eIF-4F is not intact. If exon DE-containing mRNAs, like those containing the poliovirus IRES, can be translated

in mammalian cells independent of an intact eIF-4F, then intact dicistronic 5' CAT/exon DE/LUC 3' mRNA should be associated with polysomes in poliovirus-infected mammalian cells.

COS cells were transfected with pSV_ACAT/exons DE/LUC containing an RNA hairpin upstream of CAT. At 48 hr after transfection, cells were either mock infected or infected with poliovirus. Four hours later, some of the cells from both treatments were pulse labeled with [^{35}S]methionine, and the labeled proteins were analyzed in SDS–polyacrylamide gels to confirm the efficiency of the viral infection. Translation of cellular mRNAs was drastically inhibited in infected cells, and only known viral polypeptides [Rueckert and Wimmer 1984] were synthesized during the labeling period, indicating that cap-independent translation of mRNAs could be selectively monitored at that time (not shown). Polysomal fractions were prepared from the unlabeled remainder of the cells. The optical density profile of extracts prepared from mock-infected and poliovirus-infected lysates after

chromatography on Bio-Gel A15m resin (Calzone et al. 1982) was used to analyze polysomes (Fig. 5A). Polyadenylate-containing mRNA was isolated from polysomal fraction A and nonpolysomal fraction B (Calzone et al. 1982), separated on a formaldehyde-containing agarose gel, and transferred to nitrocellulose. Two identical nitrocellulose blots were prepared and incubated individually with radioactive RNA probes complementary to CAT and LUC, respectively. As can be seen in Figure 5B,

both CAT and LUC RNA probes hybridized to a similar-size transcript, ~3700 nucleotides in length, demonstrating that the polysomal RNA fraction was intact. In mock-infected lysates, most of the dicistronic mRNA was associated with polysomes (Fig 5B, fraction A, lanes M). In lysates from poliovirus-infected cells, most of the dicistronic mRNA was again present in the polysomal fractions. Association of intact dicistronic mRNA containing both CAT and LUC sequences with polysomes in

Figure 5. Association of dicistronic mRNAs with polysomes in mock-infected and poliovirus-infected COS cells. (A) Optical density profiles of fractions obtained from separation of cellular lysates by Bio-Gel A15m chromatography (Calzone et al. 1982). Both polysomal (fraction A) and nonpolysomal fractions (fraction B), from mock-infected (□) and poliovirus-infected (◆) lysates are indicated. (B) Northern blot analysis of mRNAs isolated from fractions A and B after Bio-Gel A15m chromatography. Polyadenylate-containing RNA was separated on an agarose gel and transferred to nitrocellulose paper. Two blots were prepared and hybridized with uniformly ³²P-labeled antisense CAT and antisense LUC probes. The positions of unlabeled 18S (1900 nucleotides) and 28S (4800 nucleotides) rRNA, present in the original agarose gel, are indicated. (C) Ribonuclease protection analysis. Polysomal RNA was analyzed in denaturing polyacrylamide gels. An autoradiograph of such a gel is shown. (Lane 1) RNA probe; (lane 2) RNA probe after ribonuclease treatment; (lane 3) RNA probe hybridized to mRNA from polysomal fraction A (B; see I); (lane 4), RNA probe hybridized to RNA from pSV_A exon DE/LUC-transfected lysates, containing monocistronic DE-LUC RNA; (lane 5) RNA probe hybridized to total RNA isolated from mock-transfected COS cells. Lanes 6-8 display the position of in vitro-synthesized, labeled RNA molecules of known length. Sizes of RNAs are indicated in nucleotides.

poliovirus-infected cells is a strong indication that the second LUC cistron was translated independently of the first CAT cistron.

To provide further evidence that the polysome-associated dicistronic mRNA was intact, the integrity of the sequences between the translational termination codon in CAT and the translation initiation codon in LUC was examined by a ribonuclease protection assay, by use of the RNA probe shown in Figure 4A. An ~635-nucleotide-long RNA was protected from nuclease degradation by hybridization to mRNA from fraction A (Fig. 5C, lane 3), demonstrating that the polysome-associated dicistronic mRNA was intact. Furthermore, no protection was observed when mRNAs from nontransfected cells were used in the protection assay (lane 5). Thus, functionally dicistronic mRNA was associated with polysomes under conditions in which the bulk of cellular mRNA was not translated. Although COS cells are not a normal cell line for *Antp* gene expression, it is clear from these experiments that exon DE sequences can direct translation of the second cistron, independent of the translation of the first cistron, in dicistronic RNAs.

Discussion

Sequences in exon D of the *Antp* gene of *D. melanogaster* were shown to mediate initiation of translation by providing an IRES in cultured SL2 cells. Translation of LUC from dicistronic mRNAs, in which the LUC-coding region was the second cistron, was demonstrated by enzymatic assays following DNA transfection (Table 1) and RNA transfection (Table 2). Several lines of evidence indicate that the 5' NCR of *Antp P2* mRNA mediates internal ribosome binding, as opposed to promoting readthrough of ribosomes from the CAT to the LUC cistron in cultured cells. First, dicistronic mRNAs containing the 157-nucleotide-long exon E as an ICS did not mediate translation of the second cistron (Table 2). Because exon E is smaller than combined exons DE and is devoid of AUG triplets, one would predict enhanced rather than blocked translation of the second cistron by a termination/reinitiation event. Second, uncapped dicistronic mRNAs supported translation of the second, but not the first, cistron, demonstrating the independent translation of the two cistrons. A termination-reinitiation mechanism would predict a coupled translation of both cistrons. Furthermore, that uncapped dicistronic mRNAs failed to be translated to produce CAT suggests that the translation of LUC was unlikely to be mediated by a scanning mechanism operating on broken dicistronic mRNAs within the cell. A third and most important line of evidence for internal ribosome binding is that intact dicistronic 5' CAT/exon DE/LUC 3' mRNAs containing an RNA hairpin upstream of CAT were associated with polysomes in poliovirus-infected cells, substantiating the fact that the second LUC cistron could be translated independently of the first CAT cistron. Thus, the data are not consistent with a model in which sequences present in exons D (but not in E) facilitated efficient ribosomal readthrough from the first to the sec-

ond cistron. Instead, sequences in exon D confer to RNAs the ability to be translated by internal ribosome binding.

The 5'-terminal sequences of noncoding exon D are highly conserved between Drosophila species

Noncoding exon D is sufficient to mediate internal ribosome binding. The sequence is present in transcripts initiated at both the P1 and P2 promoters, indicating that all *Antp* mRNAs could use an internal ribosome-binding mechanism for translational initiation. The first 55 nucleotides of exon D (252 nucleotides) are highly conserved among *D. melanogaster*, *D. virilis*, and *D. subobscura* (Hooper et al. 1992). In contrast, the sequences flanking this element are highly variable. The common ancestor of *D. melanogaster* and *D. virilis* is estimated to have existed 60 million years ago (Beverley and Wilson 1982), and the ancestor of *D. melanogaster* and *D. subobscura*, 20–50 million years ago. The sequences may have been conserved because they serve important functions in regulating transcription or translation. Preliminary experiments have shown that the first 55 nucleotides of exon D can mediate internal ribosome binding when placed into the ICS region of dicistronic mRNAs (S.-K. OH and P. Sarnow, unpubl.), indicating that this 55-nucleotide sequence is one of the smallest IRES elements reported so far.

Many Drosophila mRNAs contain complex 5' NCRs

We would like to determine whether translational initiation by internal ribosome binding may be commonly used in *Drosophila* mRNAs. Up to 20% (38 of 192; Cavener and Ray 1991; D.R. Cavener, pers. comm.) of identified *Drosophila* cDNAs predict long 5' NCRs with multiple upstream AUG codons. In contrast, only 5–10% of vertebrate mRNAs have upstream AUG codons (Kozak 1989, 1991). Interestingly, vertebrate mRNAs with unusual 5' NCRs belong predominately to genes involved in growth control (Kozak 1991), indicating that certain mRNAs, whose translation products are involved in modulating growth and development, may be subject to translational control. Most *Drosophila* genes have been cloned on the basis of mutant phenotypes, suggesting that many are regulators. Regulatory genes may be subject to a more precise regulation than, for example, housekeeping genes.

Several features of the nucleotide sequences of key regulatory *Drosophila* mRNAs with long 5' NCRs are listed in Table 3. The transcription initiation sites for the mRNAs shown have been determined, excluding the possibility that the cDNA sequence was incomplete. Most of these 5' NCRs contain hundreds of nucleotides, in contrast to the 40- to 80-nucleotide-long leader of most *Drosophila* mRNAs; other exceptions are the 110- to 250-nucleotide 5' NCRs of heat shock mRNAs (Ingolia and Craig 1981). Furthermore, most of the noncoding regions summarized in Table 3, contain multiple upstream AUG codons, many of them located within se-

Table 3. Structural feature of 5'-leader sequences of *Drosophila* mRNAs

Embryonic mRNA	Length (nucleotides)	Upstream AUGs	Consensus AUGs ^a	Stop codons	References
<i>Abdominal-B</i> (pH189)	2800	31	13	108	DeLorenzi et al. (1988)
<i>Abdominal-B</i> (pH200)	494	1	0	22	DeLorenzi et al. (1988)
<i>Antennapedia</i> P1	1512	8	2	78	Laughon et al. (1986)
					Stroeher et al. (1986)
<i>Antennapedia</i> P2	1727	15	6	72	Laughon et al. (1986)
					Stroeher et al. (1986)
<i>bicoid</i>	171	2	1	5	Berleth et al. (1988)
<i>caudal</i> (maternal)	275	3	2	11	Mlodzik et al. (1987)
<i>caudal</i> (zygotic)	461	4	2	21	Mlodzik et al. (1987)
<i>Deformed</i>	490	4	0	23	Regulski et al. (1987)
<i>E74 A</i>	1891	17	4	95	Burtis et al. (1990)
<i>E74 B</i>	794	6	4	34	Burtis et al. (1990)
<i>fushi tarazu</i>	120	1	0	3	Laughon and Scott (1984)
<i>hunchback</i> (3.2 kb transcript)	511	1	0	13	Tautz et al. (1987)
<i>Krüppel</i>	185	2	2	9	Rosenberg et al. (1986)
<i>labial</i>	239	0	0	11	Diederich et al. (1989)
					Mlodzik et al. (1988)
<i>nanos</i>	262	1	0	11	Wang and Lehmann (1991)
<i>Notch</i>	799	7	1	21	Kidd et al. (1986)
<i>Sex combs reduced</i>	626	5	1	23	Lemotte et al. (1989)
<i>sevenless</i>	824	10	4	22	Bowtell et al. (1988)
<i>terminus</i>	155	0	0	6	Baldarelli et al. (1988)
<i>Ultrabithorax</i>	965	2	0	47	Kornfeld et al. (1989)

The 5' ends of the mRNAs have been determined experimentally.

^a*Drosophila* consensus sequence for translation initiation is C/A⁺AA⁺/C AUGN (Cavener 1987).

quences that are predicted to be optimal for translational start sites in *Drosophila* (Cavener 1987). Again, this raises the question of how these mRNAs can be translated efficiently according to the scanning model of translation initiation (Kozak 1989, 1991), if they are indeed translated efficiently. Recently, we have found that the 5' NCR of *Ultrabithorax* (Kornfeld et al. 1989) can also confer internal ribosome binding to a heterologous mRNA in cultured cells (S. Hoover, A. Rudie, and P. Sarnow, unpubl.). That the 5' NCRs of two homeotic mRNAs can initiate translation by internal ribosome binding raises the possibility that this mechanism may be commonly used in *Drosophila*.

Translational initiation by internal ribosome binding in *Drosophila*

Transcriptional regulation of the zygotic genome at the cellular blastoderm stage has been studied extensively. In contrast, only a few examples of translational regulation of early embryonic mRNAs have been reported (Macdonald and Struhl 1986; Mlodzik and Gehring 1987), with the exception of translational control in heat-shocked *Drosophila* embryos (Lindquist and Craig 1988; Maroto and Sierra 1988).

It is not known how many maternal and early zygotic mRNAs are translated before and during the cellular blastoderm stage. At these times, the embryo is still a single-cell syncytium that contains many rapidly divid-

ing nuclei. Translation by the cap-dependent scanning model is known to be inhibited during mitosis in mammalian cells as a result of the underphosphorylation of the cap-binding protein eIF-4E (Bonneau and Sonenberg 1987; Huang and Schneider 1991). If this is also the case in *Drosophila* cells, it is not clear how early embryonic *Drosophila* mRNAs, such as *bicoid* or *nanos*, can be translated efficiently in the syncytial embryo. A cap-independent translational mechanism could, in principle, be used by mRNAs that need to be translated during mitosis or at times when the cap-binding protein complex is not functional (Bonneau and Sonenberg 1987; Huang and Schneider 1991). One mechanism of cap-independent translation is translation by internal ribosome binding.

Is there any evidence for post-transcriptional control of *Antp* expression? It has been observed that *Antp* mRNA and protein do not always appear at the same time during embryonic development (Carroll 1986; Birmingham 1989), implying at least some post-transcriptional regulation. Such translational regulation could be under the control of factors that may be under temporal control themselves. To test whether internal ribosome binding occurs in the fly, we are in the process of constructing transgenic flies expressing dicistronic mRNAs containing exons D and E as an ICS. Monitoring the protein production of the first and second cistron, respectively, will reveal whether this mechanism is used in the organism.

Materials and methods

Cell culture and transfections

D. melanogaster SL2 cells [Schneider 1972] were grown in Schneider's *Drosophila* medium supplemented with 17% fetal bovine serum. SL2 cells were passaged every 2–3 days and maintained at a density of 1×10^6 to 8×10^6 cells/ml at 25°C. COS-1 cells (obtained from Robert Schneider, New York University, New York) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine serum. Wild-type Mahoney type-1 poliovirus was isolated from HeLa cells transfected with a pSV2 poliovirus plasmid as described [Simoes and Sarnow 1991].

Cells were transfected with 5 µg of plasmid DNA per plate with calcium phosphate, as described [Ausubel et al. 1989], except that the "glycerol shock" was omitted for SL2 cells. Cytoplasmic cell extracts were prepared 48 hr after transfection, and CAT [Ausubel et al. 1989] and LUC [De Wet et al. 1987] activity were measured in the same extracts. The amount of acetylated chloramphenicol was measured by computing densitometry (Molecular Dynamics, Sunnyvale, CA) of X-ray films. CAT activity is displayed as the percent of [14 C]chloramphenicol conversion to monoacetylated chloramphenicol.

Transfection of SL2 cells with RNA was initiated by mixing 10 µg of in vitro-synthesized, m⁷GpppG-capped RNA with a lipofectin solution [Bethesda Research Laboratories]. The mixture was then added to cells as described [Simoes and Sarnow 1991]. Extracts were prepared 19 hr after transfection, and CAT and LUC activities were measured.

Construction of plasmids

The construction of plasmid pSV_ACAT/ICS/LUC has been described [Macejak et al. 1990]. Plasmid pSV_ACAT/AP₂/LUC contains a cDNA of the entire 1730-bp 5'-noncoding region (see Fig. 1) of *Antp* P2 mRNA inserted between the CAT- and LUC-coding region. Briefly, the 5'-noncoding region of *Antp* P2, containing *Sall* and *NcoI* restriction sites at the 5' and 3' termini, respectively, was obtained by polymerase chain reaction [Ausubel et al. 1989]. The YE10 cDNA of *Antp* [Laughon et al. 1986] was used as template for the amplification reaction. The *Antp* P2 cDNA was then ligated by the *Sall* and *NcoI* sites at the termini of the amplified fragment into the ICS region of pSV_ACAT/ICS/LUC (Fig. 2A). An ATG codon provided by sequences derived from the *NcoI* restriction site was used to initiate translation of LUC. Plasmid pSV_ACAT/exon DE/LUC contains a 448-bp *Clal*-*NcoI* fragment spanning exons D and E [Laughon et al. 1986] in the ICS region. pSV_ACAT/exon ED/LUC, containing exons DE in inverted orientation in the ICS, was constructed by first repairing the ends of the 448-bp *Clal*-*NcoI* fragment with Klenow enzyme. The blunt-ended fragment was then ligated to pSV_ACAT/ICS/LUC, which had been linearized with *Sall*, and the ends were repaired with Klenow polymerase. Insertion of the *Clal*-*NcoI* fragment in one orientation produced pSV_ACAT/exon ED/LUC. Insertion of the fragment in the other orientation produced pSV_ACAT/exon DE + AUG/LUC, containing an ATG codon 36 bp upstream of the initiation codon used to express LUC. A derivative of pSV_ACAT/exon DE/LUC was constructed that contained an RNA hairpin in the 5'-noncoding region of CAT [Macejak and Sarnow 1991]. Plasmid pSV_ACAT/exon D/LUC was obtained by deleting a 180-bp *Afl*_{III}-*NcoI* fragment (exon E) from pSV_ACAT/exon DE/LUC. Following digestion with *Afl*_{III} and *NcoI*, the ends of the large vector fragment were repaired with Klenow and recircularized. Plasmid pSV_ACAT/exon E/LUC was constructed by de-

leting the *Sall*-*Afl*_{III}₁₅₅₀ fragment from pSV_ACAT/AP₂/LUC. Following digestion with *Sall* and *Afl*_{III}, the single-stranded ends of the large vector fragment were removed with mung bean nuclease and the plasmid was recircularized. Plasmid pSV_ACAT/exon C/LUC was obtained by deleting a *Clal*-*NcoI* fragment (see above) from plasmid pSV_ACAT/AP₂/LUC. In addition, plasmids were constructed that contained the promoter for T7 RNA polymerase upstream of the CAT cistron. First, a 45-bp fragment containing 30 adjacent adenosine residues was excised from pSP64 [poly(A)] (Promega) and ligated into plasmid pGEM-4 (Promega) at unique *SmaI* and *EcoRI* sites to create pGEM-4 (A_n) (constructed by L. Najita, UCHSC, Denver). To obtain T7-LUC (A_n) (constructed by S. Hambidge, UCHSC, Denver), the 296-bp *XbaI*-*NheI* fragment from pGEM-4 (A_n) was ligated into the *XbaI*-*NheI* sites located downstream of the LUC-coding region in pGEM-LUC [Macejak et al. 1990]. Finally, plasmid T7 CAT/ICS/LUC was obtained by replacing the *HindIII*-*BstEII* fragment from T7-LUC (A_n) with *HindIII*-*BstEII* fragments from various pSV_ACAT/ICS/LUC plasmids. In vitro-synthesized RNAs were purified in low-melting agarose gels in some experiments to exclude the possibility that broken RNAs were transfected into cells. Transfection into SL2 cells of purified and nonpurified RNAs yielded similar amounts of LUC, excluding the possibility of promoter-like sequences in exon D.

Isolation and analysis of RNA

Polysome-associated RNA was isolated from COS cells that were transfected with plasmid pSV_ACAT/exon DE/LUC containing an RNA hairpin upstream of the CAT-coding region. Cells were mock infected or infected with poliovirus (m.o.i. of 100) at 48 hr after transfection, as described [Simoes and Sarnow 1991]. Four hours later, cycloheximide was added to the culture medium (100 µg/ml) for 15 min to inhibit ribosome movement, lysates were prepared, and polysomes were separated from the nonpolysomal fraction by chromatography on Bio-Gel A15m as described [Calzone et al. 1982]. Polyadenylate-containing mRNAs from each fraction were isolated by oligo(dT) affinity chromatography and analyzed by Northern blot or by a ribonuclease protection assay [Zinn et al. 1983].

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