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Alternative splicing generates two distinct *Eip28/29* gene transcripts in *Drosophila* Kc cells

(ecdysone-responsive polypeptides/gene organization)

ROBERT A. SCHULZ*, LUCY CHERBAS†, AND PETER CHERBAS†‡

Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

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ABSTRACT **The *Drosophila Eip28/29* gene encodes two primary translation products, ecdysone-inducible polypeptide (EIP) 28III and EIP 29III. When cells of the Kc cell line are treated with the steroid hormone ecdysone, the number of *Eip28/29* transcripts and the synthesis of the various forms of EIP 28 and 29 increase rapidly. We have reported the sequence of the *Eip28/29* gene and of its major transcript. Here we describe a minor or short-form transcript that is about 25% of the total *Eip28/29* gene transcripts in both untreated and hormone-treated cells. This transcript is formed by the use of an alternative splice donor sequence 12 nucleotides upstream from the major donor site at the end of the second exon. Evidently the relative abundance of the two products is not hormonally regulated. The short form translation product should lack only an internal dibasic tetrapeptide. The long and short forms probably represent distinct mRNAs for EIP 28III and EIP 29III, respectively.**

We have described the overall structure and sequence of the *Drosophila melanogaster Eip28/29* gene (1). The *Eip28/29* gene is one of the genes encoding a small set of ecdysone-inducible polypeptides (EIPs), which are distinguished by their rapid induction in some *Drosophila* cell lines following ecdysone treatment (2, 3). The *Eip* genes are of interest because their induction may represent part of the initial response of these cells to the steroid hormone ecdysone.

This may be illustrated by the case of the *Eip28/29* gene. Its transcription unit (in Kc cells) is pictured in Fig. 4. It extends 2146 nucleotides (nt), includes three introns, and gives rise to a predominant mature transcript of 979 nt, which, with poly(A) added, migrates as a diffuse band of about 1200 nt (R.A.S., unpublished data). This transcript accumulates rapidly following hormone addition, increasing 5- to 15-fold and becoming about 1% of the poly(A)$^+$ RNA by the end of 3 hr (4, 5). This induction is due to an earlier rapid increase in the synthesis of *Eip28/29* gene transcripts; the transcriptional increase is detectable within 10 min and is half completed in about 30 min (5). Thus, with respect to its regulation by ecdysone, the *Eip28/29* gene may be considered a Kc cell analog to the well-known early puffs in salivary gland chromosomes (6).

In this report we consider the transcripts derived from the *Eip28/29* gene in Kc cells in greater detail. The polypeptide products of these transcripts have been described (4). These include at least six forms of EIPs 28 and 29. There are three forms of EIP 28 (I–III), whose size was estimated to be ≈28.1 kDa and which differ *inter se* by a unit charge; and there are three forms of EIP 29 (I–III) of ≈28.9 kDa, also differing *inter se* by a unit charge, but being two charges more acidic than their EIP 28 counterparts. In cells and in translation reactions aggregate EIP 28 synthesis is about three times aggregate EIP 29 synthesis, and the pattern of the products is unaffected by ecdysone, all species being induced equally (4).

RNAs, selected by hybridization to any of a number of probes derived from the *Eip28/29* gene or from cDNAs, translate to yield the entire array of polypeptide products (1, 4). However, we suspect that only the most basic form of EIP 28 (EIP 28III) and the corresponding EIP 29 species are primary translation products because, in translation reactions in which acetylation has been inhibited, these accumulate at the expense of the more acidic forms (4, 5). Still we are left to explain the production of at least two translational products, EIP 28III and EIP 29III.

Our earlier experiments have eliminated several of the possible explanations for this result. We know from hybridizations to genomic DNA that the *Eip28/29* gene is present as a single copy (1). Moreover, hybrid-selected RNA from flies homozygous for the *Eip28/29* region of chromosome 3L directs the synthesis of all forms of EIP 28 and EIP 29 (1). Hence these are not simply the alternative products of distinct alleles. Similarly cells transformed with a cloned *Eip28/29* gene overproduce all forms of both EIP 28 and EIP 29 (L.C., R. Moss, and M. M. D. Koehler, unpublished results). Sequences from four cDNA clones yielded a composite cDNA sequence used to define the transcription unit, and both S1 and primer extension experiments indicated the presence of a single 5′ terminus (1). We have been unable to distinguish discrete EIP 28 and 29 mRNAs by their stabilities as hybrids with cDNAs or by length heterogeneity following enzymatic deadenylylation (4). Thus we can entertain two remaining explanations for the origins of EIP 28 and EIP 29: (*i*) There exist distinct EIP 28 and EIP 29 mRNAs but they differ only slightly in sequence, or (*ii*) there is a single EIP 28/29 mRNA that generates distinct products by as yet undefined translational or post-translational events.

Here we report that there exist two distinct *Eip28/29* transcripts that differ internally by the presence or absence of a 12-nt sequence. These arise by the use of alternative splice donor sites. The properties of the two transcripts are consistent with the idea that they are distinct EIP 28 and EIP 29 mRNAs.

MATERIALS AND METHODS

Cells and RNAs. All RNAs were prepared from Kc cells, clone A3A11 (1, 2). In the present context "ecdysone-treated" means cells incubated 4 hr with 1 μM 20-hydroxyecdysone; this regimen elicits maximal induction of the

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Abbreviations: EIP, ecdysone-inducible polypeptide; nt, nucleotide(s).

*Present address: Department of Biochemistry and Molecular Biology, University of Texas System Cancer Center, Houston, TX 77030.

†Present address: Program in Molecular, Cellular and Developmental Biology and Department of Biology, Indiana University, Bloomington, IN 47405.

‡To whom reprint requests should be addressed.

Eip28/29 transcripts (5) and of EIP 28 and EIP 29 synthesis (3). Our procedures for cell culture and hormone treatment have been described (2, 3, 7). Total RNA and the poly(A)$^+$ fraction thereof were isolated (4). Poly(A)$^+$ RNA from adult flies homozygous for chromosome 3 was prepared (1).

DNA Clones. pKc252, the first Eip28/29 cDNA clone to be isolated, was selected from a cDNA library derived from ecdysone-treated Kc cells (4). Additional cDNA clones were isolated as described in the text.

S1-Nuclease Protection. (*i*) *Hin*fI probes: The cDNA insert of pKc45 includes a 599-base pair (bp) *Pst* I fragment (1). In the 3′-to-5′ direction of the template strand, this fragment includes a 14-nt tail added in cloning followed by a 585-nt mRNA sequence (mRNA bases 59–643, inclusive). [Wherever specified, mRNA coordinates refer to the sequence reported (1).] The *Pst* I fragment was cut with *Hin*fI and then 5′ end-labeled. Fig. 1*A* shows the relevant template strand fragments in the resulting mixture. (*ii*) *Dde* I probe: A 418-bp *Dde* I fragment was excised from the *Pst* I fragment of pKc45 and isolated. After 3′ end-labeling with Klenow fragment (8), the resulting 421-nt probe corresponds to bases 116–536 of the mRNA sequence. (*iii*) General: Probes were denatured at 70°C for 10 min, then hybridized to RNAs in 80% (vol/vol) formamide/400 mM NaCl/40 mM Pipes, pH 6.4/1 mM EDTA for 16 hr at 42°C. Nucleic acids were recovered by ethanol precipitation. The mixture was digested with nuclease S1 (Boehringer Mannheim, 10,000 units/ml) for 60 min at 37°C in 30 mM NaOAc, pH 4.5/0.25 M NaCl/1 mM $ZnSO_4$/5% (vol/vol) glycerol. Protected DNAs were displayed on 0.4-mm thick sequencing gels containing 8% polyacrylamide

(9). The 5′-end-labeled *Hpa* II fragments of pBR322 were run in parallel as markers.

Primer Extensions. As a primer specific for exon β, we selected a 30-bp *Sau*3A1 fragment representing, in the template strand, bases 320–349 of the mRNA sequence. The long *Pst* I fragment of pKc45 was digested with *Sau*3A1; the products were 5′ end-labeled, then separated on an 8% polyacrylamide sequencing gel; and the primer was isolated. This primer was hybridized with RNA at 23°C in the same hybridization solution used for S1-protection analysis. Hybrids were recovered by ethanol precipitation and dissolved in 100 mM Tris·HCl, pH 8.3, at 42°C/10 mM $MgCl_2$/148 mM KCl/100 mM each dATP, dTTP, dCTP, and dGTP/30 mM 2-mercaptoethanol. After a 5-min preincubation at 42°C, reverse transcriptase was added (final concentration 100 units/ml), and the mixture was incubated at 42°C for 1 hr. Finally the reaction was terminated by adding EDTA to 10 mM. The extended transcripts were precipitated with ethanol and analyzed on a 4% polyacrylamide sequencing gel. Reverse transcriptase was a gift of T. Papas (National Institutes of Health).

DNA Sequencing. Fragments to be sequenced were subcloned in M13mp8 (10) and sequenced by the chain-termination method (11).

RESULTS

Two Distinct *Eip28/29* Gene Transcripts Accumulate in Kc Cells. Fig. 4 summarizes our previous work on *Eip28/29* transcription in Kc cells (1). The upper diagram shows a mature transcript which, anticipating results about to be

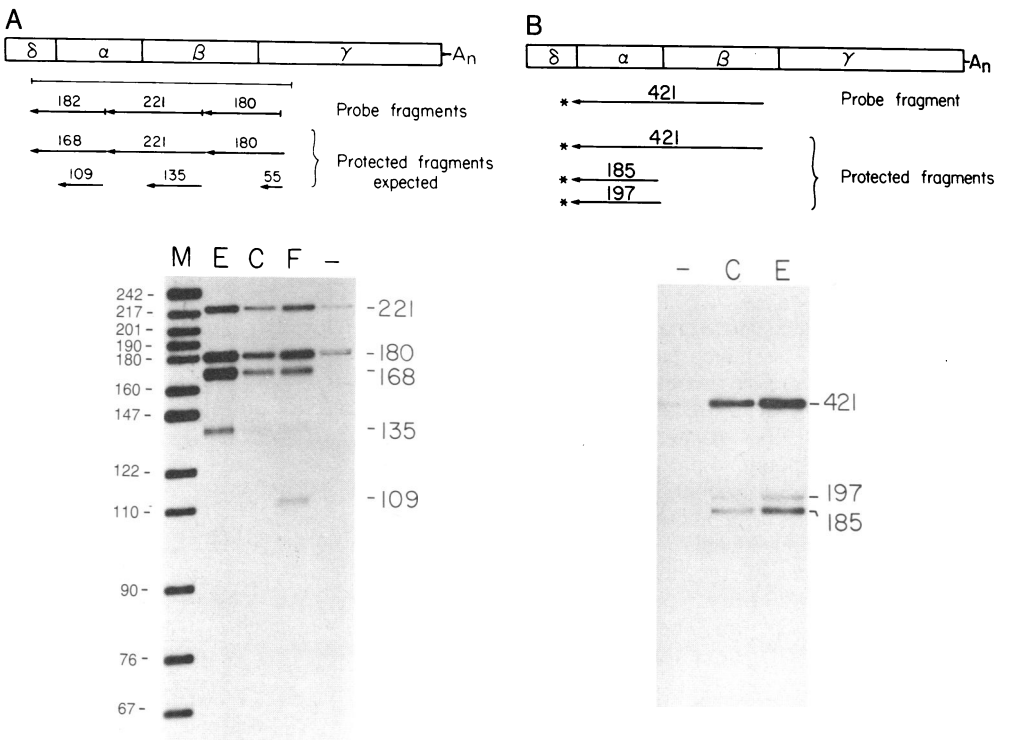

FIG. 1. Analysis of splice junctions by S1-nuclease protection. (*A*) The 5′-end-labeled *Hin*fI probe. The diagram at the top shows the structure of the predominant *Eip28/29* transcript (1), indicating the positions and extents of material derived from each of the four exons. The thinner line shows the extent of the long *Pst* I fragment from pKc45. Below it are the three probe fragments derived from the template strand. Next are the protected fragments expected in the case of a transcript spliced as is pKc45 and below that the shorter fragments that might result from variant splice junctions. RNAs were hybridized to the mixed probe, and the hybrids were digested with S1. Sizes of the protected fragments (in nt, at the right) were estimated by comparison with marker fragments in lane M. RNA samples were as follows: lane C, 1 μg of poly(A)$^+$ RNA from control cells; lane E, 1 μg of poly(A)$^+$ RNA from ecdysone-treated cells; lane F, 10 μg of poly(A)$^+$ RNA from ry^{+5} flies, homozygous for chromosome 3 (1); and lane –, no RNA. (*B*) The 3′-end-labeled *Dde* I probe. The diagram shows the position and extent of the 3′-labeled probe fragment and the positions and extents of the protected fragments observed. Sizes were estimated from marker fragments in an adjacent lane (data not shown). Lanes are labeled as in part *A*.

presented, has been labeled the long form. Its existence has been inferred from three lines of evidence: (*i*) A composite sequence derived from four cDNAs identified the genomic locations of bases 60 to the end. (*ii*) Primer extensions and S1 experiments identified a single 5′ end of the transcript corresponding to the start of exon δ. (*iii*) Simple protection experiments designed to measure the lengths of the exons yielded results compatible with the long-form structure. Here we test whether there exist minor *Eip28/29* transcripts having different structures.

We have based our probes on the large *Pst* I fragment of pKc45 (1). This fragment derives from all four exons and can, therefore, be used in protection experiments to monitor splicing at all three known junctions. In the first of the protection experiments (Fig. 1*A*), the *Pst* I fragment was cut with *Hin*fI and 5′ end-labeled. This generates three probe fragments whose lengths and positions are shown in the figure. The long form transcript is expected to protect 168 nt of the 182-nt probe; the 14 nt on the 3′ end are cloning relics. The same transcript should protect the entire 221-nt and 180-nt probe fragments. Under our experimental conditions we have not experienced problems distinguishing these protected full-length fragments from reannealed probe; however, since the probe is not present in large excess, these experiments give only a semi-quantitative measure of induction.

Poly(A)$^+$ RNAs from control and ecdysone-treated cells were hybridized to the *Hin*fI probe mixture, the hybrids were digested with S1, and the protected bands were analyzed as shown in Fig. 1*A*. As expected, these include three inducible species of 168, 180, and 221 nt. In addition a fragment of 135 nt is clearly protected and inducible. This fragment might be generated by protection of the 221-nt probe from the label to the α/β junction, suggesting that it represents RNA in which β is not normally spliced to α. Its intensity, compared to that of the 221-nt band, suggests that the unspliced or variantly spliced RNA represents about 25% of the total transcripts. We have performed similar experiments with a set of probes generated by digestion of the *Pst* I fragment with *Hpa* II and 5′ end-labeling. These experiments (not shown) suggest a similar conclusion: namely, that about 25% of the inducible RNA covering exon β is not spliced to α.

A similar protection experiment designed to analyze sequences spliced to the 3′ end of exon α is illustrated in Fig. 1*B*. The 421-base-pair *Dde* I fragment of pKc45 was 3′ end-labeled, hybridized to RNA, and treated with S1 nuclease: three protected bands were observed. The major band (421 nt) corresponds to protection of the complete probe fragment by the inducible long-form RNA. A very weak band at 197 nt may correspond to unspliced precursor RNA. However, a third protected species of ≈185 nt corresponds to protection by an RNA containing exon α that is less by about 12 nt at the 3′-terminus. We estimate this species to be about 25% of the total in both control and ecdysone-treated cells.

These results might be explained by the presence of a minor species, a short-form *Eip28/29* transcript, generated by alternative splicing at the α/β junction. The short form could be created by a splice linking a noncanonical donor site 12 nt upstream of the end of α (i.e., at the end of α_s, see Fig. 4) to β, and it should represent ≈25% of the *Eip28/29* transcript.

We note here that, whatever the origin of the 135-nt band in Fig. 1*A*, it does not signify a second *Eip28/29* allele. Lane 3 of Fig. 1*A* shows bands protected by poly(A)$^+$ RNA from flies homozygous for chromosome 3. Such RNA protects all the bands present in Kc cells, although it is possible that the 135-nt band is relatively underrepresented in flies. RNA from adult flies also protects a species of 109 nt not found in Kc cells. This band probably reflects events at the δ/α junction; we suspect it represents the existence of an alternative form of exon δ in some fly tissue(s), though it could be detecting simply an unexpectedly high concentration of unspliced precursor.

Kc Cells Contain Two *Eip28/29* RNAs Differing in Length by 12 nt. The protection experiments just described suggest the existence of a short-form transcript 12 nt shorter than the previously defined long form, the entire difference being due to differing forms of exon α. To determine whether such RNAs exist, we performed a primer extension experiment. The primer was a DNA fragment derived from exon β and representing, in the template strand, bases 320–349 of the mRNA. This fragment was isolated, labeled at its 5′ end, then hybridized to poly(A)$^+$ RNAs from control and hormone-treated cells, and extended using reverse transcriptase. Fig. 2 shows the resulting extended cDNAs. The hormone-inducible bands of 349 and 346 nt correspond to our expectations for cDNAs extended on long-form RNA. That is, previous extensions from exon α yielded an approximately equimolar mixture of products differing by ≈3 nt corresponding to full length and full length minus 3 nt (1). Plainly, in the present instance these are accompanied by an analogous doublet of inducible bands at 337 and 334 nt, i.e., ≈12 nt shorter. These are the bands to be anticipated for cDNAs extended on short-form RNA and, as expected, they represent about 25% of the total.

This result confirms the existence of two *Eip28/29* RNAs and shows that they differ in length by ≈12 nt, the difference deriving from sequences upstream of base 320.

A Short-Form cDNA Sequence Confirms the Structure. If the preceding inferences are correct, our cDNA library prepared from Kc cells should contain two classes of Eip28/29 clones differing in sequence at the α/β splice junction. Therefore, we isolated and characterized 27 Eip28/29 cDNA clones from our initial library (4); our survey included our original Eip28/29 cDNA clone pKc252 and several clones isolated in a screen using pKc252 insert as probe (1). It also included a third group of clones that share the property of covering the α/β splice junction and that were identified by rescreening the same cDNA library selecting

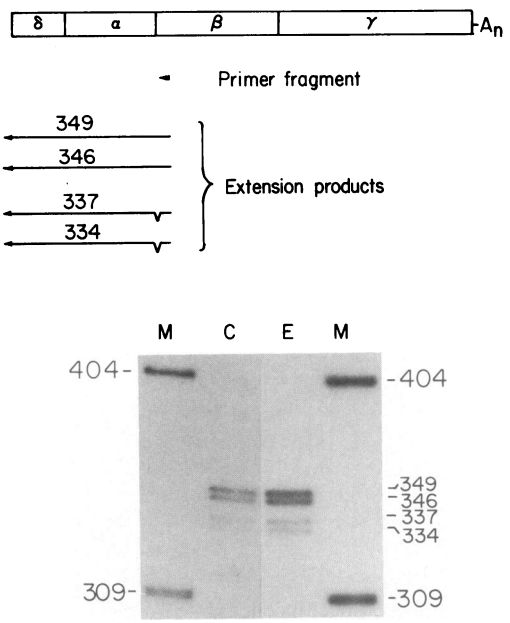

FIG. 2. Primer extension reveals two *Eip28/29* transcripts. The diagram shows the position of the primer within exon β above our interpretation of the resulting extended fragments. Poly(A)$^+$ RNAs from control (lane C) and hormone-treated (lane E) cells were hybridized to the primer that was extended using reverse transcriptase. Fragment lengths were determined by comparison to the migration of *Hpa* II-digested pBR322 fragments (lane M). Lanes C and E were from different experiments.

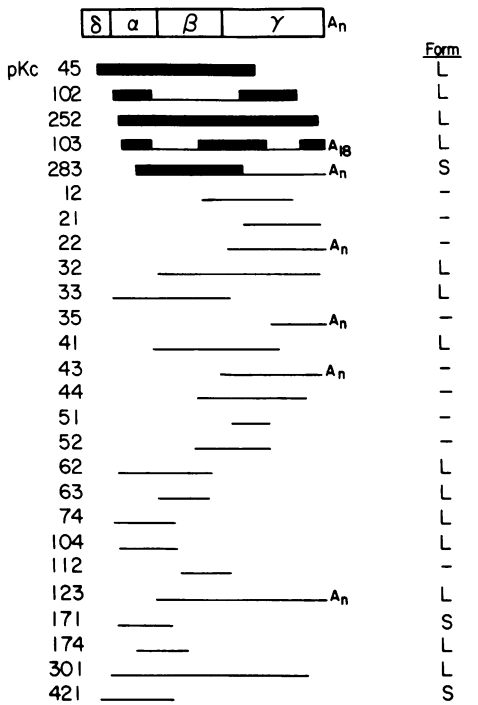

FIG. 3. Characterization of Eip28/29 cDNA clones. The predominant, long-form transcript is shown. Below are indicated the positions and extents of each cDNA insert we have characterized. Heavy lines indicate regions that have been sequenced, either previously (1) or, in the case of pKc283, as part of the present work. Each insert that includes the α/β splice junction has been classified as either long (L) form or short (S) form.

colonies that hybridize to each of two genomic *Bam*HI fragments whose junction falls within the α/β intron.

All 27 clones were partially mapped, with special attention to sites for the enzyme *Rsa* I. There are six sites for *Rsa* I in the long-form sequence (see figure 2 in ref. 1), one site being defined by the initial four bases of the 12-nt region that differentiates long form from short form. Our survey revealed one clone that corresponds to a partially processed transcript, the β/γ but not the α/β and δ/α introns having been excised. The remaining 26 clones are illustrated in Fig. 3. Seventeen of these include the α/β splice region. Of the latter, 14 were diagnosed as long form by their *Rsa* I maps, while the remaining 3 appeared to be short-form clones. Save for this dimorphism, the maps of all the cloned segments appeared to be portions of an invariant composite.

pKc283, one of the short-form clones, was sequenced in the region of the α/β splice (Fig. 3). The sequence of pKc283 is precisely that suggested by the S1 protection and primer-extension results and is shown in Fig. 4. Thus a minor form of *Eip28/29* transcript exists. It represents about 25% of the total *Eip28/29* transcript in both control and ecdysone-treated cells and is apparently formed by the use of an alternative donor site 12 nt upstream from the end of exon α. The result is the formation of two distinct mRNAs differing in length by 12 nt and in coding potential by 4 codons.

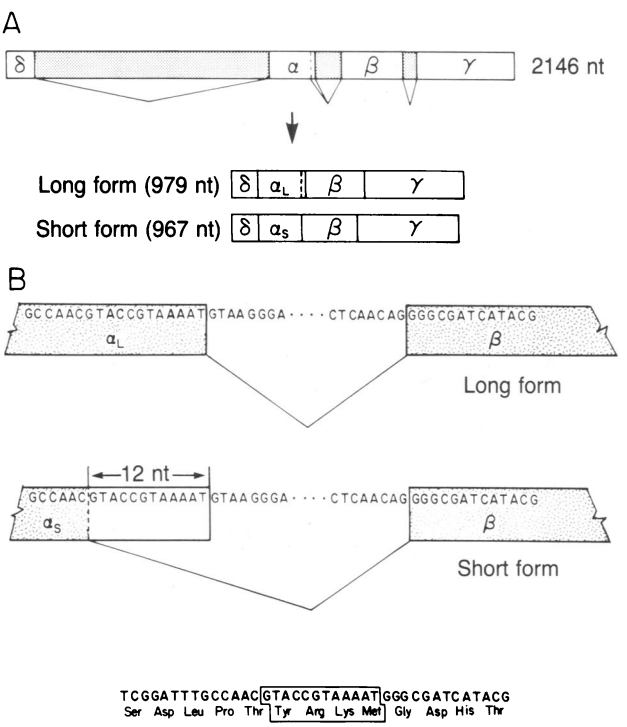

FIG. 4. Splicing patterns at the *Eip28/29* gene. (*A*) Origins of the long- and short-form transcripts by the use of the donors α_L and α_S. (*B*) Long- and short-form sequences in the vicinity of the α/β splice junction. α_S lacks the last 12 nt of α_L and generates an mRNA with a 4-codon deletion.

DISCUSSION

Our results demonstrate that *Eip28/29* transcripts are spliced to yield two alternative stable species, a predominant long form of 979 nt and a less abundant short form of 967 nt. None of our observations have detected any additional heterogeneity in the population of mature transcripts. Thus we have mapped and/or sequenced about 12 transcript equivalents of cDNAs without finding evidence for further length heterogeneity. RNAs that have exon δ start at a site unaffected by ecdysone (1), and (in Kc cells) all RNAs that have exon α have α spliced to δ (Fig. 1*A*) and to β (Fig. 1*B*). All those RNAs that include β are spliced to either α_L or α_S corresponding to the long and short forms, respectively (Figs. 1*A* and 2). Finally, all RNAs that include exon γ have γ spliced to β (Fig. 1*A*). Our experiments might miss a subclass in which β is spliced to an alternative γ. And, despite the fact that we have mapped six cDNAs that include polyadenylylation sites (including one short-form cDNA), we cannot exclude the existence of a minor class using a different poly(A) site or sites. Our previous efforts to detect heterogeneous *Eip28/29* transcripts (4) by either length or hybrid stability were unsuccessful; now we can interpret those results as a failure to distinguish between two RNA species that differ by only 1% in length.

The preceding is an accurate description of the situation in Kc cells. However, we note that it may not apply to all the tissues or stages of flies. In particular, we note that adult RNA includes a moderately abundant transcript that probably contains α not spliced to δ. While it is possible that this arises by unusually slow excision of the δ/α intron, we think it more likely that this transcript reflects the tissue-specific use of one or more variant δ exons.

While it is unambiguous that Kc cells contain two mature *Eip28/29* transcripts, our results do not address directly the identities of these transcripts as mRNAs. Still the properties of the long- and short-form transcripts are strongly suggestive that they encode the EIP 28III and EIP 29III, respectively. (*i*) The ratio of aggregate EIP 28 synthesis to aggregate EIP 29 synthesis is ≈3 and is unaffected by ecdysone (3–5). Similarly observations reported here suggest that the ratio of long-form RNA to short-form is ≈3 and remains so during the substantial induction of both. Quantitative measurements (5) of the relative abundances of the two forms during induction support this conclusion. (*ii*) The two transcripts, like the two classes of polypeptides (1), are produced by a single *Eip28/29* allele. (*iii*) EIP 28III is two charges more basic than EIP 29III

(A. Bieber, M. M. D. Koehler, and P.C., unpublished data). Fig. 4 shows that use of α_S not only maintains the reading frame of the mRNA but should generate a polypeptide lacking only the tetrapeptide Tyr-Arg-Lys-Met. Thus it is reasonable to suppose that the short-form transcript serves as a functional mRNA and yields a primary product two charges more acidic than that of the long form.

There is one obvious discrepancy in this interpretation. The sizes of the forms of EIP 28 and 29 were estimated from their mobilities in $NaDodSO_4$/polyacrylamide gels as 28.1 and 28.9 kDa, respectively (3); the polypeptide products encoded by the long- and short-form transcripts ought to be 28,218 and 27,640 Da, respectively. Thus if we are correct, EIP 29 is 2% smaller rather than 2% larger than EIP 28. We discount this discrepancy because we suspect that an anomaly in migration of this magnitude is not unreasonable in a comparison of two virtually identical polypeptides differing by two charges (or ≈8% of the calculated net charge at pH 10).

In short, while positive identification of the products of the long- and short-form transcripts must await further work, it seems likely that their existence accounts for the production of both EIP 28III and EIP 29III by the unique *Eip28/29* gene.

Thus *Eip28/29* is one of the numerous transcriptional units known to produce multiple products. While many of these cases involve choices among promoter and/or polyadenylylation sites, *Eip28/29* is an example of that class of genes in which one primary transcript yields various spliced products by the use of alternative donor and/or acceptor sites (12, 13). In some of these cases the result is restricted to noncoding regions; for example, the use of two donors 24 nt apart in the 5′-noncoding region of rat calcitonin (14). But in others, as in *Eip28/29*, the differential splice leads to polymorphism at the protein level. Thus, about 10% of chicken ovomucoid lacks an internal Val-Ser dipeptide because of the use of alternate donor sites 6 nt apart (15). Adenovirus-2 E1A 13S and 12S RNAs result from the use of alternative donors and yield polypeptides differing internally by 46 residues; the shorter polypeptide may lack the ability to activate 13S transcription (13). In human growth hormone mRNA, the use of alternative acceptor sites leads to forms differing by 15 amino acids; the shorter form lacks the insulin-like activity of the longer (16). In rat prolactin mRNA a similar event produces polypeptides differing by one alanine residue (17). Numerous more complex examples, some involving the facultative expression of whole exons or introns have been described (12).

In the case of *Eip28/29* it appears likely that the ratio of the two mature transcripts reflects the relative frequencies of splicing at α_L and α_S. This will be true if, as seems plausible, all the long- and short-form intermediates have comparable stabilities. Indeed, judged by their accumulation rates following ecdysone addition, the two forms do not differ significantly in their stabilities in hormone-treated cells (5). Thus, we may take it as a reasonable working hypothesis that α_L functions as a donor about three times as well as α_S. A corollary is that ecdysone does not influence the choice of splicing pathway.

We can say little about the actual mechanism of donor site selection. The consensus donor sequence is usually taken to be (MAG$^{\nabla}$GTRAGT (18), where M stands for adenine or cytosine, and ∇ indicates the site of splicing. The α_L sequence AAT$^{\nabla}$GTAAGG, like the donor sequences at the ends of δ and β (1), represents an adequate match to the consensus. By contrast, the α_S sequence AAC$^{\nabla}$GTACCG represents a poor match and should hybridize poorly to U1 snRNA (12). Thus, restricting ourselves to the five critical nucleotides following the junction, GTACC occurs only rarely, in 2/139 normal junctions catalogued by Mount (18), and in two cryptic donor junctions observed in mutant globins (19, 20). However, it may be that α_S benefits from the reported competitive advantage of upstream donor sites (12). Finally, Solnick (21) has suggested that the use of alternative processing pathways may reflect the existence of competing secondary structures in the primary transcript. In this case one might look for a structure hindering the use of α_L. We have searched the α/β intron for sequences capable of forming stable hairpins with α_L, but without success. However, if when α_L is blocked α_S is used efficiently, such secondary structures might differ by <1 kcal/mol. While it is possible to identify potentially paired structures that might influence such an equilibrium, it is difficult to evaluate their significance.

The EIPs and their genes were identified solely on the basis of their regulatory properties, i.e., their rapid induction by ecdysone. Clearly until we know the functions of the EIPs 28/29, we can say little about the significance of their sequence similarities and differences. Still it is interesting that the biosynthetic pathway described here creates two polypeptides that differ only by the internal tetrapeptide Tyr-Arg-Lys-Met. Proteolytic processing of polypeptide precursors often occurs at pairs of basic residues (22). Thus it is possible that EIP 28, but not EIP 29, is cleaved to yield N- and C-terminal fragments of ≈8.5 and 20 kDa, respectively. Some preliminary evidence suggests that this processing pathway exists (5).

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