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att, a Target for Regulation by *tra2* in the Testes of *Drosophila melanogaster*, Encodes Alternative RNAs and Alternative Proteins

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We have identified a gene, *alternative testis transcripts* (*att*), which is alternatively expressed, at both the RNA and protein levels, in testes and somatic tissues. The testis-specific RNA differs from somatic RNAs in both promoter usage and RNA processing and is dependent on the function of the *transformer 2* gene. The differences between the somatic and testis RNAs have substantial consequences at the protein level. The somatic RNAs encode a protein with homology to the mammalian Graves' disease carrier proteins. The testis RNA lacks the initiation codons used in somatic tissue and encodes two different proteins. One of these begins in a testis-specific exon, uses a reading frame different from that for the somatic protein, and is completely novel. The other protein initiates translation in the frame of the somatic RNA at a Leu CUG codon which is within the open reading frame for the somatic protein. This produces a novel truncated version of the Graves' disease carrier protein-like protein that lacks all sequences N terminal to the first transmembrane domain.

The genes of the sex determination regulatory cascade of *Drosophila melanogaster* affect all aspects of sex determination and sexual differentiation, including overt sexual differentiation, appropriate sexual behavior, and male and female fertility (see references 6, 11, 12, 22, 23, 33, 34, 37, and 44 for some of many reviews). *transformer 2* (*tra2*) is a key gene acting in the pathways controlling both somatic sexual differentiation and male fertility (7). In somatic tissues the tra2 protein, in conjunction with the female-specific transformer (tra) protein, regulates sex-specific splicing of *doublesex* (*dsx*) RNA (21, 24, 38, 42, 52, 53). In addition to regulating *dsx*, *tra2*, again in conjunction with *tra*, also regulates at least one other gene that controls sexual behavior and sex-specific nervous system differentiation (47, 48, 50).

tra2 is also necessary for male germ line function, although not for sex determination of the male germ line. Mutant males carrying loss-of-function *tra2* mutations have morphologically normal germ lines but display incomplete spermatogenesis and produce immotile sperm (7). Two examples of *tra2* regulation in the male germ line are known. Each exhibits both germ line-specific promoter usage and germ line-specific differences in RNA processing. One of these targets is *tra2* itself. The tra2 protein regulates its own level by inhibiting the productive splicing of its own pre-mRNA (31). The *exuperantia* (*exu*) gene also shows a dependence on *tra2* in the male germ line. In this case the requirement for *tra2* is not absolute. Instead, the presence of $tra2^+$ seems to increase the efficiency of male germ line-specific processing events, including alternative splicing and polyadenylation (20).

The product of the *tra2* gene in *D. melanogaster* is a known RNA-binding protein (2, 3, 19, 21, 51, 52). This binding depends on a single ribonucleoprotein motif in the central region of the protein. On *dsx* RNA, tra2 binds to a 300-bp region containing six copies of a 13-nucleotide motif. This region is essential both molecularly and biochemically for proper female activation of the regulated splice site by *tra* and *tra2*, and the repeats have been shown to be tra2 binding sites (2, 3, 10, 21, 24, 39, 42, 51, 52).

We are interested in understanding the way in which the sex differentiation cascade controls terminal differentiation and function, particularly in those as yet unidentified genes which might be in the pathway controlling sex-specific behaviors and the pathway controlling male fertility. One gene functioning in both pathways is *tra2*. In this paper we report the isolation of one such downstream target of *tra2* regulation. The gene, *att*, is differentially expressed in the soma and the male germ line in a *tra2*-dependent manner. Regulation involves both tissue-specific promoter choice and differential polyadenylation site choice. The germ line-specific, *tra2*-dependent RNAs encode novel proteins from each of two reading frames.

MATERIALS AND METHODS

***Drosophila* culture and strains.** *D. melanogaster* were grown on standard cornmeal-molasses-yeast-agar medium as described previously. Mutations and chromosomes are as described in the work of Lindsley and Zimm (27) or the references therein or as noted below. Hybridization to polytene chromosomes was performed as previously described (35).

Cloning methods and molecular biology. Most procedures were performed as described by Sambrook et al. (43).

Phage clones that hybridized to a ^{32}P-labeled oligonucleotide probe (5′-TCT TCAATCAACA-3′) designed to represent the best-conserved *dsx* 13-nucleotide repeat were isolated from a *D. melanogaster* λ EMBL3 genome library by plaque hybridization after transfer to nitrocellulose membranes (Schleicher & Schuell). Hybridization was carried out in BLOTTO (6× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA {pH 7.7}], 5% nonfat dried milk) at room temperature (approximately 22°C), and washes were performed in 6× SSPE at room temperature until the background signal was reduced. Phage were plaque purified, and high-titer stocks were prepared by plate lysis. Restriction digests of phage DNA were probed with the *dsx* oligonucleotide in order to identify fragments of interest for Northern (RNA) analysis. The cDNA libraries used were constructed from imaginal discs (λgt10 library, kindly provided by Tso-Pang Yao) and testes (λZAP library, kindly provided by Tulle Hazelrigg) and were screened with genomic probes.

Genomic clones and cDNAs were sequenced after cloning into Bluescript (Stratagene) and generating nested deletions with exonuclease III and S1 (Promega Erase-a-Base System, manufacturer's protocol). Sequencing was by the dideoxy chain termination method using the Sequenase Kit (United States Bio-

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chemical; manufacturer's protocol). Sequence analysis and assembly made use of the Sequencher, DNA Star, and DNA Strider (29) packages. Sequence comparisons were carried out by the BLAST network service at National Center for Biotechnology Information (NCBI) (1) and the FASTA program (16) run remotely either at NCBI or the European Molecular Biology Laboratory.

In vitro transcription and translation of cDNAs was performed in reticulocyte lysates using the TNT translation kit from Promega. Translations using [^{35}S]methionine used an otherwise Met-free reaction mix. Translations using [^{35}S]cysteine and [^{35}S]methionine used an otherwise Cys-free reaction mix.

RNA was isolated as follows. Flies were homogenized in equal volumes of 8 M guanidinium thiocyanate and phenol chloroform (1:1). Fly parts were removed at 12,000 × *g* for 10 min. The aqueous phase was extracted with phenol-chloroform, and the nucleic acids were precipitated with ethanol. The pellets were suspended in diethyl pyrocarbonate-treated water, and an equal volume of 8 M LiCl was added. The RNA was precipitated in a microcentrifuge, suspended in diethyl pyrocarbonate-treated water, precipitated with ethanol, and resuspended in diethyl pyrocarbonate-treated water. Poly(A) RNA was isolated by batch chromatography to oligo(dT)-cellulose by standard methods.

Unless noted, 2 μg of poly(A) RNA per lane was size fractionated by electrophoresis on 1% agarose gels. The gel and running buffer were composed of 1× morpholineethanesulfonic acid (MOPS; pH 7.0) plus 6% formaldehyde. RNA was transferred by blotting with 20× SSPE to nylon membranes (Hybond; Amersham). Hybridization and washing were done under the conditions of Church and Gilbert (13). Membranes were prehybridized for 30 min in 0.5 M $NaHPO_4$ (pH 7.2)–7% sodium dodecyl sulfate (SDS)–1 mM EDTA (pH 8.0)–1% bovine serum albumin (BSA) at 65°C. Denatured probe was added directly to the prehybridization solution and allowed to hybridize to RNA for a minimum of 6 h at 65°C. Membranes were washed twice at 65°C with 40 mM $NaHPO_4$ (pH 7.2)–2% SDS–1 mM EDTA (pH 8.0)–1% BSA for 10 min each time and then twice at 65°C in 40 mM $NaHPO_4$ (pH 7.2)–0.5% SDS–1 mM EDTA (pH 8.0) for 10 min each time.

Reverse transcriptase PCR (RT-PCR) was performed using StrataScript modified Maloney murine leukemia virus RT (Stratagene, La Jolla, Calif.) for first-strand synthesis. First-strand cDNA synthesis, as well as later PCRs, was primed with an oligonucleotide complementary to positions 670 to 689 of the testis RNA, within exon 5 in the common region of the message. Second-strand synthesis, as well as later PCRs, was primed with an oligonucleotide corresponding to positions 84 to 104 of the testis RNA, within the testis-specific exon. Reaction products were size fractionated on agarose gels, blotted, and probed with a radiolabeled oligonucleotide complementary to positions 174 to 193 of the testis RNA, within exon 4, the first common exon.

RESULTS

Isolation of *att*, a downstream target of *tra2* in the testis. The goal of this study was to identify novel genes under the control of *tra2* in either somatic tissues or the male germ line. As part of one screen we identified a candidate genomic clone, F8P5, with characteristics of a potential *tra2* target.

As an initial test to determine if F8P5 encoded a potential target gene of the sex determination cascade, a 2.4-kb *Eco*RI restriction fragment of F8P5 was used to probe a Northern blot of male and female RNA (Fig. 1, left). Three polyadenylated transcripts were identified. Two of the transcripts (1.8 and 1.6 kb) appear in both males and females, and the third (1.4 kb) is male specific. The presence of a sex-specific transcript is consistent with the possibility that F8P5 contains a target of the sex determination cascade.

In order to determine if the RNAs derived from F8P5 were dependent on *tra2* and if they were under the control of the somatic sex differentiation cascade or of *tra2* function in the male germ line, RNA was isolated from flies that were mutant for *tra*, *tra2*, or *dsx* and from flies that lack a germ line as a result of being offspring of homozygous *tudor* mothers (Fig. 1, right). *dsx*-independent targets of the somatic sex differentiation cascade are expected to show a dependence of their female RNA pattern on the function of *tra* and *tra2* but not on the function of *dsx* or the presence of a male germ line. Genes downstream of *dsx* are expected to show a dependence on *tra*, *tra2*, and *dsx*. Targets of *tra2* in the male germ line are expected to show a male RNA pattern that is dependent on the presence of a male germ line and on the function of *tra2* in males. It should be noted that XX; *tra* pseudomales and XX; *tra2* pseudomales as well as XX and XY; *dsx* animals have rudimentary gonads. Although a small amount of male germ line differentiation occurs in such animals, they are not expected to express male germ line RNAs at levels approaching what is seen in the wild type (40).

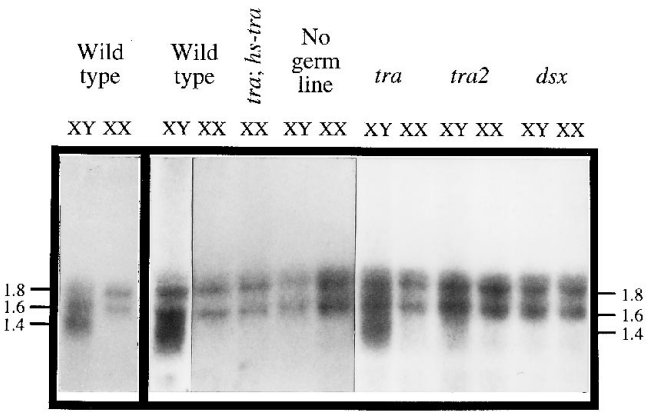

FIG. 1. *att* encodes a male germ line-specific, *tra2*-dependent RNA. (Left) Poly(A)$^+$ RNA from wild-type males and females was size fractionated and blotted to nitrocellulose prior to probing with the 2.4-kb *Eco*RI restriction fragment of F8P5. Two bands common to males and females as well as a 1.4-kb male-specific band can be seen. (Right) Poly(A)$^+$ RNA from XX and XY flies of the genotypes indicated was treated as on the left. Wild-type RNA was from Canton-S; *hs-tra* indicates RNA from female flies who have a heat shock-driven *tra* female cDNA as their only *tra* allele (36). Such flies exhibit certain abnormalities of sexual behavior consistent with a partially male nervous system (reference 49 and our unpublished observations). "No germ line" flies were the progeny of homozygous *tudor* mothers. *tra* flies carried tra^1/Df(3L)stj7. *tra2* flies were homozygous for $tra2^B$. *dsx* flies were homozygous for dsx^1. The three RNA bands and their approximate sizes (in kilobases) are indicated. All lanes are from a single autoradiogram.

As shown in Fig. 1 (right), the male transcript was not detected at high levels in XX pseudomales homozygous for either *tra* or *tra2*. This indicates that somatic maleness alone is not sufficient for the appearance of the male transcript and that *tra* and *tra2* do not play a role in suppression of this transcript in females. This pattern of hybridization is inconsistent with F8P5 containing a female somatic target of the sex determination cascade. Figure 1 also shows that the male-specific transcript is not present, or is present at low levels, in male flies lacking a germ line or mutant for *tra2*. Mutations in *tra* had no effect on the presence of this transcript in males. This pattern of expression is completely consistent with our predictions for a *tra2* target in the male germ line. As predicted for an RNA expressed in testes, flies mutant for *dsx* do not produce the male-specific transcript at high levels. More-sensitive tests using RT-PCR (not shown) detect male-type RNAs in XX; tra^- and in dsx^- individuals. Consistent with the low level of male germ line development in these animals (40), the level of male RNA is much lower than is seen in the wild type. Neither lack of a germ line nor mutations in *tra*, *tra2*, or *dsx* had any effect on the non-sex-specific transcripts, consistent with the idea that these RNAs are expressed in somatic tissues and are not under the control of the sex determination cascade.

In order to verify the tissue distribution of the male-specific transcript, we have examined expression in the testes, accessory glands, and remaining somatic tissues of dissected males (Fig. 2A). These results show that the male transcript is indeed in the testes. The results also demonstrate that this RNA is the major RNA variant in testes and that it is present at a very low level or absent in somatic tissues. The data in Fig. 2A, as well as information from intact animals, suggest that the testis-specific RNA is substantially more abundant in testes than the somatic RNAs are in the tissues which express them.

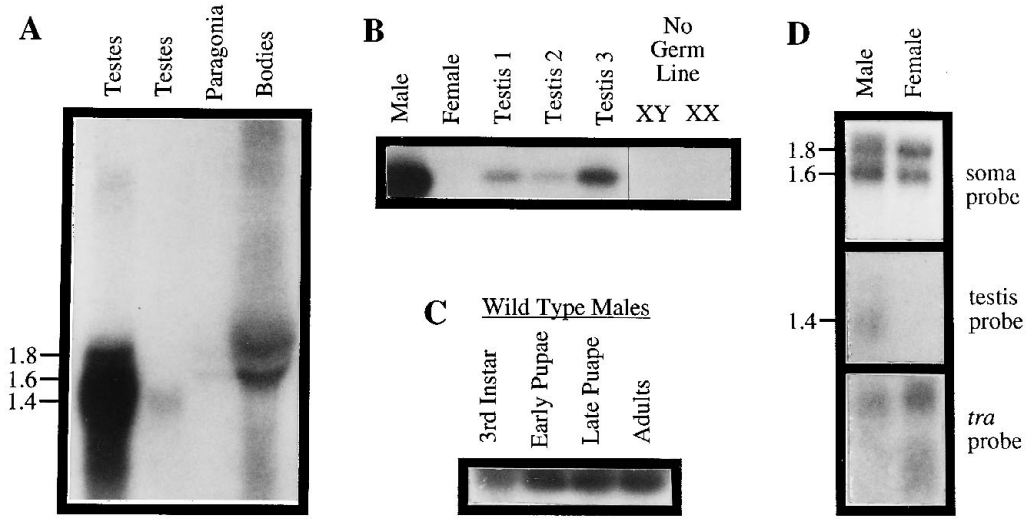

FIG. 2. *att* expression patterns. (A) An *att* cDNA was used as a probe against a blot of total RNAs derived from the testes of 10 males, the paragonia of the same 10 males, or the remaining bodies of the same 10 males. The second testis lane was obtained in a shorter exposure of the first testis lane. As judged by hybridization to a gene for a ribosomal protein (*rp49*), the lanes with testes and paragonia have equal amounts of RNA while the lane with bodies has eight times more RNA. (B) The testis RNA is expressed early in sperm development. RT-PCR using male-specific and common region primers was used to assay *att* expression in males, females, flies with no germ line, and three separate portions of the testis. Testis 1 contains approximately the first 10% of the testis, testis 2 contains the region from approximately 10 to 25% of the length of the testis, and testis 3 contains the rest of the testis. (C) Time course of male *att* expression. RT-PCR was used to assay expression of the testis-specific RNA in wild-type male third-instar larvae, early pupae, late pupae, and adults. Female or no-germ-line controls run at the same time show no signal (data not shown). (D) Message-specific exons from the 5′ ends of cDNA clones were used to probe male and female RNA. The probes are as described in the text. The testis probe contains approximately 35 nucleotides of the first common exon as well as the testis-specific portion. The bottom panel is a reprobing of the blot originally probed with the testis probe, using a *transformer* cDNA to reveal the presence of RNA and to verify the sex of the individuals that were the source of the RNA in the male and female lanes. Sizes are indicated on the left in kilobases.

It is possible that the *tra2* effect on F8P5 expression results from *tra2*$^-$ germ cells stopping differentiation prior to the time at which F8P5 is expressed. This seems unlikely in that *tra2*$^-$ germ cells arrest at the nuclear condensation stage (nuclei condense but go no further), after meiosis and well after bulk transcription has ceased (7, 18). To test this, we examined the time and position of F8P5 expression.

Most transcription in the male germ line occurs during the G_2 phase of the cell cycle in primary spermatocytes, prior to meiosis (18). Little transcription occurs prior to this stage or after it. Because of the architecture of the testis, these stages are spatially separated (26). Stem cells, mitotic cells, and G_1 and S primary spermatocytes are located near the apical tip of the testis (within the first 10% of the testis length); primary spermatocytes in the G_2 growth phase are located just beyond this region (up to about 25 to 30% of the length of the testis); and meiotic and postmeiotic nuclei are located farther along the testis. Testes were dissected into three fractions: testis 1 covered approximately the first 10% of the testis, testis 2 covered from 10 to 25%, and testis 3 covered the rest. Testis 1 may have contained some G_2 cells from the growth region but did not reach the postmeiotic region. RNA from each sample was then subjected to RT-PCR using primers to detect the male transcript (Fig. 2B). A clear signal is seen in all three fractions, consistent with transcription in premeiotic G_2 with carryover of message into the postmeiotic stages.

Because of the way in which sperm differentiate, it is a formal possibility that some late-transcribed RNAs could be carried into the most apical region of the testis by the elongating sperm tail. To control for this possibility, we have used a second test based on the developmental time course of testis differentiation and of F8P5 expression. Male germ line function begins by the third-instar larval stage, but the developing cells do not reach the meiotic stage until the pupal period (18). Thus, expression of F8P5 testis RNA during the larval period indicates that F8P5 expression occurs prior to meiosis and therefore prior to the *tra2* block in differentiation. To test this, we used RT-PCR of RNA from wild-type male third-instar larvae, early and late pupae, and adults (Fig. 2C). These results clearly show F8P5 testis RNA expression in third-instar larvae and thus demonstrate that F8P5 testis expression occurs prior to the *tra2* arrest point.

Thus, we conclude that the 2.4-kb fragment identified in our screen encodes one RNA that is *tra2* dependent in the male germ line. It also encodes two RNAs expressed in the somatic tissues of both males and females. On the basis of the differential expression patterns between the germ line and soma and the dependence on *tra2* function, we have named this gene *att* for *alternative testis transcript*.

The testis-specific RNA is a structurally distinct variant of the somatic RNAs. To examine the differences between the three transcripts, we isolated and characterized cDNAs that represent these RNAs. Three classes of cDNAs, 1.8 and 1.6 kb (somatic class) and 1.4 kb (testis class), were identified. Only the 1.4-kb class was recovered from the testis library (20 clones). The three cDNA species hybridize to each other and to all three transcripts identified with the 2.4-kb *Eco*RI genomic fragment (not shown). No additional transcripts have been detected as coming from the 2.4-kb R1 fragment, either on RNA blots probed with the region of the 2.4-kb fragment distal to *att* or in the multiple cDNA screens with the 2.4-kb fragment as a probe.

Initial restriction analysis revealed that the testis and somatic cDNAs share a common core region, with the testis clones being shorter than some somatic clones at both the 5′ and 3′ ends. Fine-structure restriction analysis and DNA sequencing (see below) localized putative testis- and soma-specific regions to the 5′ ends of the DNAs, as well as revealing a

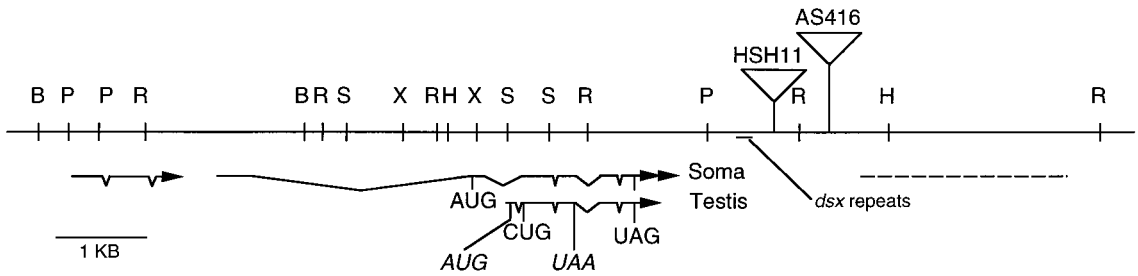

FIG. 3. A map of the *att* region. In situ hybridization and DNA blotting place *att* in salivary chromosome region 92E3/4, between the right-hand breakpoints of Df(3R)oF4 and Df(3R)oB16 (27). The restriction map in the immediate region of *att* is shown. Restriction sites are indicated by single letters (R, *Eco*RI; S, *Sal*I; H, *Hin*dIII; B, *Bam*HI; X, *Xho*I; P, *Pst*I). Transcripts and splicing patterns are indicated below the restriction map. The transcript to the right of *att* has not been mapped with regard to direction or introns. AUG in the somatic message indicates the first ATG in the frame of the GDC protein. The CUG indicates the potential CUG start codon in the GDC protein frame of the testis RNA. Both GDC proteins terminate at the UAG codon indicated under the testis message. The AUG in the testis-specific exon indicates the start of the alternative reading frame associated with that message, while UAA indicates the end of the alternative reading frame (see Fig. 4). Two transposons in the region are noted by triangles. *AS416* (14) is homzygous lethal. *HSH11* (25) has reduced viability when homozygous or in combination with a deficiency, with zygotic viability being less when the mother is heterozygous for the deficiency. *AS416*/*HSH11* heterozygotes are fully viable. The relationship between *HSH11*, *AS416*, and *att* is not clear.

soma-specific 3′ end (Fig. 3). Further analysis demonstrates that the unique 5′ regions of testis- and soma-specific cDNAs hybridize to specific transcripts. A 125-bp fragment from the 5′ end of a testis class cDNA hybridizes only to the male germ line-specific transcript, while a 300-bp fragment from the 5′ end of a somatic-class cDNA hybridizes only to the non-sex-specific transcripts (Fig. 2D). In addition, all of the somatic-class cDNAs isolated terminate at approximately the same 5′ position while the testis class cDNAs terminate at a different 5′ position. Reverse transcription followed by PCR amplification verifies that the 5′ ends of the cDNAs are at or near the 5′ ends of the RNAs (data not shown).

The hybridization patterns are consistent with alternative promoter usage or the presence of a very small common 5′ exon followed by alternative splicing. No such exon has been identified in any cDNA. Rapid amplification of cDNA ends-PCR generates fragments consistent with transcriptional initiation at or near the 5′ ends of the longest cDNAs. As part of an attempt to identify possible common 5′ exons, we isolated and partially sequenced cDNA clones for the next upstream transcription unit. This gene is transcribed toward *att* and terminates within approximately 600 bases of the first somatic exon. Use of genomic DNA fragments between the 5′ end of the somatic cDNAs and the 3′ end of the upstream gene as probes on RNA blots fails to show hybridization to RNAs with an *att* pattern or to any other RNA. RNA blotting with probes farther upstream reveals additional transcription units, but none with hybridization patterns matching that of *att.*

cDNAs have been completely sequenced, and exon-intron boundaries have been determined from sequences of genomic clones relative to cDNA clones. The complete soma- and male germ line-specific cDNA sequences are presented in Fig. 4. The transcription unit spans at least 6.8 kb. The soma-specific and male germ line-specific transcripts contain six and five exons, respectively (Fig. 3). Exons 4, 5, 6, and 7 are common to all transcripts. Exons 1 and 2 are specific to the somatic class of cDNAs, and exon 3 is specific to the male germ line RNA. Use of the germ line-specific promoter produces a transcript in which exons 1 and 2 are replaced by exon 3 in the male germ line transcript (Fig. 3). Substitution of exon 3 for exons 1 and 2 is sufficient to account for the size difference between the smaller soma-specific message (1.6 kb) and the male germ line-specific message (1.4 kb). The size difference between the two soma-specific messages results from the use of alternative polyadenylation sites as identified from poly(A)-containing cDNAs (Fig. 3 and 4). The 5′ polyadenylation site matches the consensus AATAAA and resides 250 nucleotides upstream of the more 3′ (ATTAAA) site, which is not a perfect match to the consensus. These two poly(A) sites appear to be used with equal efficiency in the soma, as the two somatic transcripts appear at equal levels. All of the testis-specific cDNAs terminate at or before the proximal poly(A) site. Northern analysis with the male germ line-specific exon demonstrates that the testis class of RNAs are polyadenylated almost exclusively at the proximal poly(A) site (Fig. 2). We conclude from these data that the *tra2*-dependent male germ line RNA shows both alternative promoter usage and alternative poly(A) site choice relative to the somatic messages.

Hybridization to genomic *att* DNA, using a *dsx* repeat 13-base oligonucleotide probe, and DNA sequencing identified two closely linked *dsx* repeat-like sequences, in the same orientation as the *att* transcripts, 3′ to both the male-specific and somatic RNAs. From these data it is unclear if the *dsx*-like repeats actually have a role in regulation of *att* by *tra2*, although they are in a position in which they might affect poly(A) site choice of nascent transcripts which continue 3′ of the final poly(A) addition site.

Alternative somatic and germ line transcripts encode structurally different proteins. To determine if the differentially expressed *att* RNAs had consequences for protein coding, we examined the open reading frames (ORFs) present in the different RNAs. The single long ORF of the somatic RNAs begins in exon 2 and continues through exon 7 (Fig. 3 and 4). This ORF is preceded by stop codons in all three reading frames and ends before the alternative poly(A) sites. Three AUG codons lie within 25 codons of each other (nucleotide positions 190, 223, and 262 of Fig. 4A). In vitro translation is consistent with use of one of these three start sites.

The testis RNA lacks exons 1 and 2 and contains instead exon 3. Exon 3 contains no start codon in the frame of the somatic message (Fig. 4B). The first AUG in this frame occurs in exon 4 at a position equivalent to amino acid 137 of the longest potential somatic protein. Non-AUG initiation sites, notably CUG and GUG codons, have been observed in *D. melanogaster* (8, 15, 45, 46). Examination of the testis RNA sequence reveals that the first CUG codon in the somatic reading frame (position 76 of the longest somatic protein) is in a reasonable context for translation initiation (AGC CUG A) (9).

The testis-specific RNA also contains a second ORF in a reading frame different from the one in the somatic RNAs (Fig. 4B). This begins with an AUG in testis-specific exon 3

A. Somatic Transcript

```
1
aag aca aag aat tag ctc gcg gtt aaa gag aat aca gca cct gtg ttt ttg gaa tcg aaa
61
atc agt gcg aaa cgc cta cca ctg ctg tgt gtt gtt gtt att gtt gcc aag gcc gaa gga
121
gcg aaa agg gag gcc gaa aaa aaa gaa tct gtg cta aca aaa aca aag gtc ccc cag tcc
181
gag tct gcg atg agc cga ctc ctg gag aag ccc aac atc gcc atg tcc atc aag tcc acg
          M   S   R   L   L   E   K   P   N   I   A   M   S   I   K   S   T
241
ggt agt cag ctc tcg agc acg atg acg aca gcc tcg gcg acg ctg tcc tcg gac ctg gac
G   S   Q   L   S   S   T   M   T   T   A   S   A   T   L   S   S   D   L   D
301
gat gcg gac aca tcc cgc acc cag ctg agt cca tcg gaa aca tct gga gta gtc ctc gta
D   A   D   T   S   R   T   Q   L   S   P   S   E   T   S   G   V   V   L   V
361
ccc gcc act aca gtc aca ccc atG CGC CAG AAA ATC GAC CAG GTG GTG ATC AGC CTG ATA
P   A   T   T   V   T   P   M   R   Q   K   I   D   Q   V   V   I   S   L   I
421
TCC GGA GCA GCG GCG GGG GCG CTG GCC AAG ACG GTC ATC GCC CCG CTG GAC CGC ACA AAG
S   G   A   A   A   G   A   L   A   K   T   V   I   A   P   L   D   R   T   K
481
ATC AAC TTC CAG ATC CGC AAC GAT GTG CCC TTC TCG TTT CGA GCC TCG CTG CGC TAC CTG
I   N   F   Q   I   R   N   D   V   P   F   S   F   R   A   S   L   R   Y   L
541
CAA AAC ACC TAT GCC AAC GAG GGC GTT TTG GCC CTG TGG CGG GGG AAC TCG CCC ACG ATG
Q   N   T   Y   A   N   E   G   V   L   A   L   W   R   G   N   S   P   T   M
601
GCC AGA ATC GTG CCC TAC GCA GCC ATA CAG TTC ACG GCC CAC GAG CAG TGG CGT CGC ATC
A   R   I   V   P   Y   A   A   I   Q   F   T   A   H   E   Q   W   R   R   I
661
CTG CAC GTC GAC AAG GAC GGC ACC AAC ACG AAA GGT CGT CGG TTT TTG GCT GGC TCC CTG
L   H   V   D   K   D   G   T   N   T   K   G   R   R   F   L   A   G   S   L
721
GCG GGA ATC ACC TCA CAG TCG CTG ACG TAT CCT CTG GAC CTG GCA CGC GCC CGC ATG GCC
A   G   I   T   S   Q   S   L   T   Y   P   L   D   L   A   R   A   R   M   A
781
GTC ACG GAT CGG TAT ACT GGC TAT CGG ACG CTG CGA CAA GTC TTC ACC aag ATC TGG GTG
V   T   D   R   Y   T   G   Y   R   T   L   R   Q   V   F   T   K   I   W   V
841
GAG GAG GGT CCG CGG ACG CTG TTC CGC GGC TAC TGG GCG ACC GTT CTC GGC GTG ATT CCC
E   E   G   P   R   T   L   F   R   G   Y   W   A   T   V   L   G   V   I   P
901
TAT GCG GGC ACC TCC TTC TTC ACC TAC GAG ACT CTT AAG CGG GAA TAC TAT GAA GTG GTC
Y   A   G   T   S   F   F   T   Y   E   T   L   K   R   E   Y   Y   E   V   V
961
GGC AAC AAT AAA CCC AAT ACT CTA GTC TCG CTG GCC TTC GGT GCT GCG GCT GGT GCC GCC
G   N   N   K   P   N   T   L   V   S   L   A   F   G   A   A   A   G   A   A
1021
GGG CAA ACG GCC AGC TAT CCA TTG GAC ATT GTG CGG CGA AGA ATG CAG ACA ATG CGG GTG
G   Q   T   A   S   Y   P   L   D   I   V   R   R   R   M   Q   T   M   R   V
1081
AAC ACG GCT GGC GGA GAT CGG TAC CCA ACC ATC CTG GAG ACT CTC GTC AAG ATC TAT CGT
N   T   A   G   G   D   R   Y   P   T   I   L   E   T   L   V   K   I   Y   R
1141
GAG GAG GGC GTC AAG AAC GGT TTC TAC AAG GGA CTT AGC ATG AAC TGG ATC AAG GGA CCC
E   E   G   V   K   N   G   F   Y   K   G   L   S   M   N   W   I   K   G   P
1201
ATC GCC GTG GGC ATC AGC TTC TCC ACC TAC GAT CTG ATC AAG GCG TGG CTC ACG GAG CTG
I   A   V   G   I   S   F   S   T   Y   D   L   I   K   A   W   L   T   E   L
1261
GCC AAC TTG AGA CGG GTT GAG AAA TAG CCC AGC TCC CTG CCC CTG CCA CTC GTC ACC CAT
A   N   L   R   R   V   E   K   *
1321
AAA CAC CAC ACC AAA AAC GCA CTG CTT TGA TCT TAA GTC TCG CGA TCG ATG GAT GAG CTG
1381
AGG AAA TAG AAG TGA AGC ACC GAC ATG CTC TAA TTG TTA GAT ATT TTT TAG CTG TTA GTT
1441
GTG AGG GAG AGA AGC GAT GAA GTG AAG ACA CCC GAA AGC GCT TAA CTT TGT TAT TAA CCG
1501
TAA TTG ATT TAT TAT CGC CGT AAG CCG AAA GTA TAT CAG CCA ATA AAC TGT CTG AAT AAT
1561
GAC TCG CCT GGC ACA TAC TGC TCC CCA GGA CCT CGT ATT CAA TGG TGT TGC TTT GAC AAT
1621
AAC ACT CCT AGA TTA ATG ATT ACG ATT TAC CCG AAC ATT TTG TAT ATT GTG TAT TGC CAA
1681
CTC AAT GTC AAG GCG CTG CGA CTG TTA AAA TAG TCA TGA ATT TGA GGG TGT TCA CAC CAG
1741
CAT ATA CTA TGT AAT TCT TGT ATG TTA GTG TGT TTT TAT TAT TAA AAT TTA CAA AAT TGT
1801
TTA ACT G
```

B. Testis Transcript

```
1
gcg aca caa tga aaa ctc ttg aat ttt tgt gtt tgt cga cag taa aat gtc gag tat ttg
61
act tca aac cga act aaa tta tag agc gta gtg gag tgt caa agt ttc cta tgt ccc tcc
                                                                  M   S   L   R
121
gtt tat tta tcc ata gtc gcg cat tcg gTG CGC CAG AAA ATC GAC CAG GTG GTG ATC AGC
  L   F   I   H   S   R   A   F   G   A   P   E   N   R   P   G   G   D   Q   P
181
CTG ATA TCC GGA GCA GCG GCG GGG GCG CTG GCC AAG ACG GTC ATC GCC CCG CTG GAC CGC
L   I   S   G   A   A   A   G   A   L   A   K   T   V   I   A   P   L   D   R
  D   I   R   S   S   G   G   G   A   G   Q   D   G   H   R   P   A   G   P   H
241
ACA AAG ATC AAC TTC CAG ATC CGC AAC GAT GTG CCC TTC TCG TTT CGA GCC TCG CTG CGC
T   K   I   N   F   Q   I   R   N   D   V   P   F   S   F   R   A   S   L   R
  K   D   Q   L   P   D   P   Q   R   C   A   L   L   V   S   S   L   A   A   L
301
TAC CTG CAA AAC ACC TAT GCC AAC GAG GGC GTT TTG GCC CTG TGG CGG GGG AAC TCG CCC
Y   L   Q   N   T   Y   A   N   E   G   V   L   A   L   W   R   G   N   S   P
  P   A   K   H   L   C   Q   R   G   R   F   G   P   V   A   G   E   L   A   H
361
ACG ATG GCC AGA ATC GTG CCC TAC GCA GCC ATA CAG TTC ACG GCC CAC GAG CAG TGG CGT
T   M   A   R   I   V   P   Y   A   A   I   Q   F   T   A   H   E   Q   W   R
  D   G   Q   N   R   A   L   R   S   H   T   V   H   G   P   R   A   V   A   S
421
CGC ATC CTG CAC GTC GAC AAG GAC GGC ACC AAC ACG AAA GGT CGT CGG TTT TTG GCT GGC
R   I   L   H   V   D   K   D   G   T   N   T   K   G   R   R   F   L   A   G
  H   P   A   R   R   Q   G   R   H   Q   H   E   R   S   S   V   F   G   W   L
481
TCC CTG GCG GGA ATC ACC TCA CAG TCG CTG ACG TAT CCT CTG GAC CTG GCA CGC GCC CGC
S   L   A   G   I   T   S   Q   S   L   T   Y   P   L   D   L   A   R   A   R
  P   G   G   N   H   L   T   V   A   D   V   S   S   G   P   G   T   R   P   H
541
ATG GCC GTC ACG GAT CGG TAT ACT GGC TAT CGG ACG CTG CGA CAA GTC TTC ACC AAG ATC
M   A   V   T   D   R   Y   T   G   Y   R   T   L   R   Q   V   F   T   K   I
  G   R   H   G   S   V   Y   W   L   S   D   A   A   T   S   L   H   Q   D   L
601
TGG GTG GAG GAG GGT CCG CGG ACG CTG TTC CGC GGC TAC TGG GCG ACC GTT CTC GGC GTG
W   V   E   E   G   P   R   T   L   F   R   G   Y   W   A   T   V   L   G   V
  G   G   G   G   S   A   D   A   V   P   R   L   L   G   D   R   S   R   R   D
661
ATT CCC TAT GCG GGC ACC TCC TTC TTC ACC TAC GAG ACT CTT AAG CGG GAA TAC TAT GAA
I   P   Y   A   G   T   S   F   F   T   Y   E   T   L   K   R   E   Y   Y   E
  S   L   C   G   H   L   L   L   H   L   R   D   S   *
721
GTG GTC GGC AAC AAT AAA CCC AAT ACT CTA GTC TCG CTG GCC TTC GGT GCT GCG GCT GGT
V   V   G   N   N   K   P   N   T   L   V   S   L   A   F   G   A   A   A   G
781
GCC GCC GGG CAA ACG GCC AGC TAT CCA TTG GAC ATT GTG CGG CGA AGA ATG CAG ACA ATG
A   A   G   Q   T   A   S   Y   P   L   D   I   V   R   R   R   M   Q   T   M
841
CGG GTG AAC ACG GCT GGC GGA GAT CGG TAC CCA ACC ATC CTG GAG ACT CTC GTC AAG ATC
R   V   N   T   A   G   G   D   R   Y   P   T   I   L   E   T   L   V   K   I
901
TAT CGT GAG GAG GGC GTC AAG AAC GGT TTC TAC AAG GGA CTT AGC ATG AAC TGG ATC AAG

Y   R   E   E   G   V   K   N   G   F   Y   K   G   L   S   M   N   W   I   K
961
GGA CCC ATC GCC GTG GGC ATC AGC TTC TCC ACC TAC GAT CTG ATC AAG GCG TGG CTC ACG
G   P   I   A   V   G   I   S   F   S   T   Y   D   L   I   K   A   W   L   T
1021
GAG CTG GCC AAC TTG AGA CGG GTT GAG AAA TAG CCC AGC TCC CTG CCC CTG CCA CTC GTC
E   L   A   N   L   R   R   V   E   K   *
1081
ACC CAT AAA CAC CAC ACC AAA AAC GCA CTG CTT TGA TCT TAA GTC TCG CGA TCG ATG GAT
1141
GAG CTG AGG AAA TAG AAG TGA AGC ACC GAC ATG CTC TAA TTG TTA GAT ATT TTT TAG CTG
1201
TTA GTT GTG AGG GAG AGA AGC GAT GAA GTG AAG ACA CCC GAA AGC GCT TAA CTT TGT TAT
1261
TAA CCG TAA TTG ATT TAT TAT CGC CGT AAG CCG AAA GTA TAT CAG CCA ATA AAC TGT CTG
1321
AAT AAT GA
```

FIG. 4. Sequence of *att*. (A) The composite sequence of the somatic cDNAs is shown as is a conceptual translation beginning at the first AUG of the longest ORF. Lowercase nucleotides are derived from the portion of the message specific to the somatic transcripts. Italicized amino acids are encoded by the soma-specific portion of the RNA. The junction between the soma-specific region (exons 1 and 2) and the common region (exons 4 to 7) occurs between bases 382 and 383. Pairs of nucleotides which are boldfaced and underlined are separated by introns in the genomic DNA. The underlined AATAAA and ATTAAA are potential polyadenylation signals, while the single underlined residues following them are sites of polyadenylation in cDNAs. The alternative sites of polyadenylation are separated by just over 240 nucleotides. Potential alternative protein start sites are marked in boldface (M). (B) Sequence of the testis-specific RNA. Lowercase nucleotides are derived from the portion of the message specific to the testis transcripts. Italicized amino acids are encoded by the testis-specific portion of the RNA. The junction between the testis-specific region (exon 3) and the common region (exons 4 to 7) occurs between bases 148 and 149. Pairs of nucleotides which are boldfaced and underlined are separated by introns in the genomic DNA. The underlined AATAAA is a potential polyadenylation signal, while the single underlined residue following is the site of polyadenylation in cDNAs. The novel ORF specific to the testis RNA begins at residue 111. The potential CUG initiation site for the ORF which is in phase with the somatic ORF starts at position 181. All methionine and cysteine residues in the two proteins are shown in large boldface underlined letters.

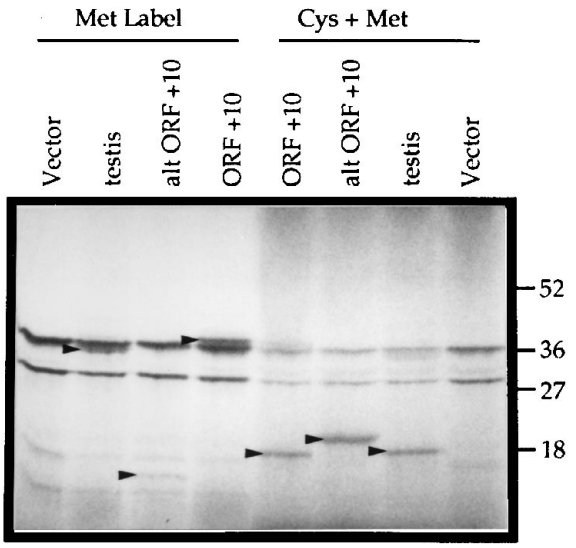

FIG. 5. Translation of *att* cDNAs. RNA copies of a male cDNA were used to prime in vitro translation in the presence of [^{35}S]Met (left) or [^{35}S]Cys plus [^{35}S]Met (right). Bio-Rad prestained molecular weight markers (positions indicated on the right in thousands) were used as standards. The vector lanes were primed with RNAs transcribed from the Bluescript pSK vector (Stratagene). altORF+10 indicates a modified version of the testis cDNA containing a 10-amino-acid addition at the end of the putative testis-specific ORF. This insert truncates the ORF shared with the somatic reading frame. ORF+10 indicates a modified version of the cDNA with a 10-amino-acid addition at the end of the ORF shared with the somatic RNAs. Arrowheads indicate RNA-dependent bands preferentially labeled by [^{35}S]Met (left) or [^{35}S]Cys (right).

and extends for nearly 200 amino acids. No sequences with high homology to the conceptual translation of this ORF are found in searches of GenBank or in the Prosite database.

The frame which overlaps the somatic ORF contains five methionine codons and no cysteine codons. The reading frame which is unique to the testis message contains only one methionine codon, the one at the start of the ORF, and three cysteine codons. Translation with [^{35}S]Met reveals a message-dependent band migrating at a position consistent with translation from the first CTG codon of the common ORF (Fig. 5). Translation with [^{35}S]Cys yields a new band of a size consistent with translation from the first AUG of the testis-specific ORF.

To verify the reading frames used for translation of the Met-containing and Cys-containing proteins, we produced modified versions of the RNAs in which a 10-codon extension was separately added to each reading frame. The extension of the testis-specific ORF adds a premature stop codon to the somatic ORF. As can be seen in Fig. 5, the expected changes in protein products occur in response to mutations extending each reading frame. Thus, we conclude that the testis-specific RNA encodes two different proteins, both of which are produced by metazoan translation machinery.

Conceptual translation of the somatic ORF and database searches show that the potential somatic *att* product has high homology (36.3% identity) to the bovine version of a major autoantigen in Graves' disease, the Graves' disease carrier (GDC) protein (54). On the basis of homology, both the att and GDC proteins are members of the mitochondrial ADP-ATP carrier class of proteins (Fig. 6) (see, for examples, references 5, 30, 41, and 54 and the references therein). The protein encoded by this reading frame of the testis-specific RNA is substantially altered and represents a novel variant within the protein family. Proteins beginning at the first testis start site (CUG) would lack all protein sequence preceding the first transmembrane domain, with initiation occurring within the first transmembrane domain.

DISCUSSION

We have isolated and characterized a new gene, *att*, whose regulation is dependent on *transformer 2* in *D. melanogaster*. This gene is regulated in a *tra2*-dependent, sex-specific manner. Three transcripts from this gene have been identified. One of these is produced in the male germ line in a *tra2*-dependent manner, while the other two are produced in the somatic tissue of both males and females, independent of genes in the sex determination hierarchy. In whole testes there is a strong bias toward the testis class of RNA, although some somatic-class RNA is observed. It is not clear if the somatic forms of the RNAs in the testes are produced in the germ line-derived cells of the testes or if they are derived from somatic cells of the testes.

There are two differences between the soma- and testis-specific RNAs: (i) the two different RNAs appear to use specific promoters, and (ii) there are message-specific differences in the usage of poly(A) addition sites. In somatic tissues a single promoter is used. Transcripts from this promoter are processed in such a way that a 5′ splice site which is used in the testis RNA is bypassed in favor of an upstream 5′ splice site used in the somatic messages. In addition, RNAs derived from the somatic promoter can be polyadenylated at either of two poly(A) addition sites, leading to two classes of somatic RNAs represented at approximately equal levels in steady-state RNA. In the testis, an alternative promoter is used. In steady-state RNA there is a strong bias toward testis RNAs which have terminated at the proximal poly(A) site. The differences between the poly(A) site representation of somatic and testis class RNAs could result from a difference in poly(A) site usage, a difference in stability of the RNAs polyadenylated at different sites for the different classes of RNA, or a combination of the two.

The differences between the somatic and testis RNAs have consequences for protein coding. The somatic RNAs encode a member of the ATP-ADP carrier class of proteins. The testis RNA cannot encode the same protein as the somatic RNAs. Instead it contains a novel ORF beginning in the testis-specific exon as well as an ORF encoding an N-terminal truncation of the ATP-ADP carrier protein.

Two other genes, *tra2* and *exuperantia* (*exu*), have been shown to be subject to expression differences based on *tra2* function in the testis. In each case there are interesting parallels to *att*. Specifically, all three genes appear to have testis-specific promoters which are different from the promoters used in other tissues, and RNAs derived from these promoters are subject to differential processing. In the case of *tra2* and *exu*, this alternative processing is based, at least in part, on the function of *tra2*. In the case of *tra2* autoregulation, *tra2* activity inhibits the splicing of one intron, leading to the production of an RNA which does not code for a functional tra2 protein in the testis (3, 4, 31, 32). In the case of *exu*, *tra2* activity is involved in specific events at the 3′ end of the RNA. Although these processing events can occur in *tra2* mutants, they occur with full efficiency only in *tra2*$^{+}$ animals. Alternative processing of the *exu* RNAs is complicated in that testis RNAs skip a polyadenylation site used in the female germ line. Instead of using this site, a testis-specific splicing event occurs within the 3′ untranslated region of the RNA, splicing out the region that contains the ovary poly(A) site. Poly(A) addition then occurs at a downstream site (20). The mechanism by which *tra2* reg-

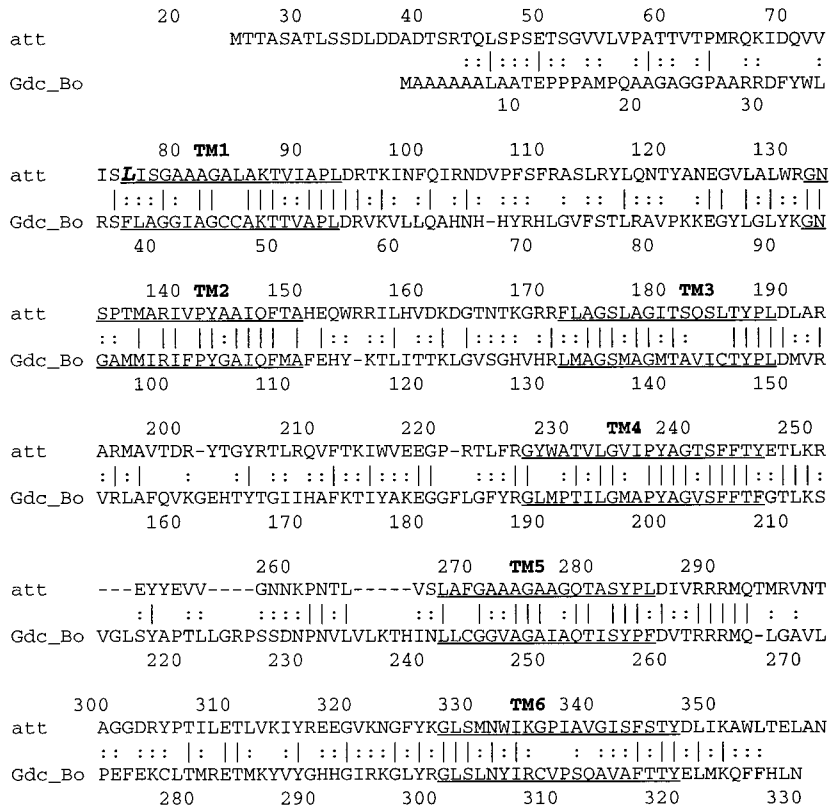

FIG. 6. Sequence comparison between att and the bovine version of the GDC protein. The att somatic protein sequence (starting from the second ATG as presented in Fig. 4A) was aligned to the bovine version of the GDC protein (Gdc_Bo) (17) by the program FASTA (16). Gdc_Bo is the protein of maximum similarity to att in databases searches. Large underlined regions marked "TM" are putative transmembrane domains on the basis of similarity to assignments made for other members of the family (5). In the region of similarity between the proteins, they show 36.3% sequence identity. In the putative transmembrane domains, there is 57% identity between the two proteins. The boldface italicized L at position 77 is the putative start site for translation in the testis RNA of the common reading frame that encodes the GDC protein-like protein. Vertical lines indicate identity; colons indicate similarity.

ulates either itself or *exu* is currently unknown (see reference 28 for a discussion).

The testis-specific *att* RNAs show both specific promoter usage and a switch to the use of only one of the two poly(A) addition sites. How might these differences from the somatic expression occur? (i) The tra2 protein could directly control promoter usage. (ii) The tra2 protein could control some primary target gene which then alters *att* promoter usage. The latter suggestion is analogous to the situation in somatic tissues in which *tra2* (with *tra*) regulates expression of the dsx transcription factor. In the case of both i and ii changes in poly(A) site usage might be envisioned as following from some context of the primary transcript or some difference between the array of processing factors in the germ line and soma. (iii) Promoter usage could depend entirely on testis-specific factors independent of *tra2*, but poly(A) site usage could be dependent on *tra2* function. This would make *att* regulation formally similar to regulation of *tra2* and *exu*. These possibilities and the role of the downstream *dsx*-like repeats in this process are currently under study.

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