

Sex-Specific Regulation of Yolk Protein Gene Expression in *Drosophila*

John M. Belote,* Alfred M. Handler,†
Mariana F. Wolfner,*‡ Kenneth J. Livak,*§
and Bruce S. Baker*

* Department of Biology, B-022
University of California, San Diego
La Jolla, California 92093

† Developmental Biology Center
University of California, Irvine
Irvine, California 92717

Summary

Many of the genes in the regulatory hierarchy controlling sex determination in *Drosophila melanogaster* are known. Here we examine how this regulatory hierarchy controls the expression of the structural genes encoding the female-specific yolk polypeptides. Temperature shift experiments with a temperature-sensitive allele of the sex determination regulatory gene *transformer-2* (*tra-2*) showed that *tra-2* function is required in the adult for both the sex-specific initiation and maintenance of YP synthesis. Control of the YP genes by this regulatory hierarchy is at the level of transcription, or transcript stability. The results of temperature shift experiments with abdomens isolated from *tra-2^{ts}* homozygotes support the notion that the *tra-2* function acts in a cell-autonomous manner to control YP synthesis. These results provide a paradigm for the way this regulatory hierarchy controls the terminal differentiation functions for sexually dimorphic development.

Introduction

Many regulatory genes responsible for sexual development in *Drosophila melanogaster* have been identified. Genetic analyses of these genes suggest that they function together as parts of a regulatory hierarchy (for review see Baker and Belote, 1983). The earliest acting genes in this hierarchy control both somatic and germ-line sex determination, as well as dosage compensation (Maroni and Plaut, 1973; Van Deusen, 1976; Cline, 1979, 1983, 1984; Lucchesi and Skripsky, 1981). After these initial steps the regulation of these processes diverges. There are four known genes (*transformer*, *tra*; *transformer-2*, *tra-2*; *intersex*, *ix*; and *doublesex*, *dsx*) that function after this divergence in a single regulatory circuit, in which the *tra* and *tra-2* products act to control the expression of the bifunctional *dsx* locus (Figure 1), to bring about sexual differentiation in somatic cells (Baker and Ridge, 1980).

Genetic studies have also established some of the ba-

sic properties of the mechanism by which this regulatory hierarchy acts during development to control the expression of the structural genes whose products are directly responsible for the sexually dimorphic phenotypes of differentiated cells. All the sex determination regulatory genes examined thus far (including *tra*, *tra-2*, and *dsx*) act in a cell-autonomous manner (i.e., none make diffusible products) (Baker and Ridge, 1980). The times during development when the products of this hierarchy have functioned sufficiently for normal sexual differentiation to occur have been investigated using temperature-sensitive (*ts*) *tra-2* mutants. In chromosomal females the absence of *tra-2* function throughout development leads to male somatic sexual differentiation. Temperature shifts with *ts tra-2* mutants showed that in cells producing the cuticular structures of the adult this regulatory gene does not determine sex at a discrete time, but rather is needed at several times within single cell lineages to control different aspects of sexual differentiation (Belote and Baker, 1982). This suggested that the different times at which functioning of this regulatory hierarchy is required reflect the different times at which the structural genes under its control are normally expressed in a cell lineage.

The present study is aimed, in part, at testing this hypothesis by examining at a molecular level how this hierarchy regulates the expression of specific structural genes. We have chosen for analysis the three genes that encode the major yolk polypeptides (YPs) (for review see Postlethwait and Jowett, 1981). These genes are normally expressed only in females producing YPs throughout adulthood by two tissues: the adult fat body cells (Gelti-Douka et al., 1974) and the ovarian follicular epithelium (Brennan et al., 1982). The YPs made in the fat body are secreted as vitellogenin into the hemolymph (Hames and Bownes, 1978; Postlethwait and Kaschnitz, 1978), from which they are sequestered by maturing oocytes (Mahowald, 1972).

All three YP genes have been cloned (Barnett et al., 1980; Hovemann et al., 1981) and studies of their expression suggest that their coordinate control occurs at the level of transcription (Barnett and Wensink, 1981; Hung et al., 1982). Two sets of *trans*-acting regulatory functions that control YP gene expression have been identified. First, YP synthesis is hormonally regulated. Both 20-hydroxyecdysone and juvenile hormone have been found to promote YP synthesis (Postlethwait and Handler, 1979), and YP transcript levels are increased in female fat body treated with either hormone (Shirk et al., 1983; Postlethwait and Shirk, personal communication). It is likely that a signal at eclosion triggers the hormonal events resulting in YP gene expression and ovarian maturation (Handler and Postlethwait, 1977). Although YP synthesis does not normally occur in males, a low level of synthesis can be stimulated by very high levels of 20-hydroxyecdysone (Bownes, 1982; Shirk et al., 1983). Second, YP synthesis is also controlled by the regulatory genes governing somatic sexual differentiation (Postlethwait et al., 1980; Bownes and Nöthiger, 1981; Ota et al., 1981). That the

‡ Present address: Section of Genetics and Development, Bradfield and Emerson Halls, Cornell University, Ithaca, New York 14853.

§ Present address: Central Research and Development Department, Experimental Station 328/367, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898.

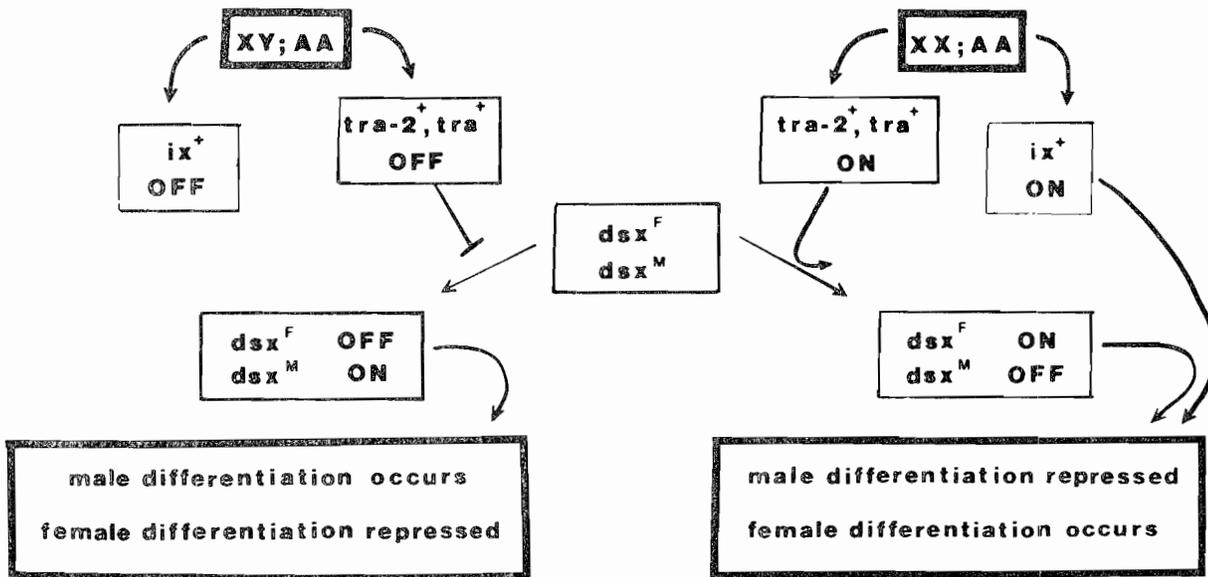


Figure 1. Model for the Roles of the *tra*⁺, *tra-2*⁺, *ix*⁺, and *dsx*⁺ Loci in Sex Determination
 The bifunctional *dsx*⁺ locus can express either of two functions: *dsx^M* is expressed in males and represses female differentiation. The *tra*⁺ and *tra-2*⁺ loci function in females to allow the expression of *dsx^F*. The *ix*⁺ locus acts in conjunction with *dsx^F* in females to repress male differentiation.

functioning of this hierarchy is necessary for the synthesis of YPs in females is shown by the observation that chromosomal females that have been transformed into males as the result of homozygosity for a null mutation at either the *tra* or *tra-2* locus do not synthesize YPs. The normal activity of this regulatory hierarchy is also required in chromosomal males to prevent YP synthesis: loss-of-function mutations at the *dsx* locus (which transform both males and females into intersexes) result in the synthesis of YPs in chromosomal males. The available data are all consistent with the view that these regulatory genes interact with one another to control YP synthesis in the same manner that they interact to control the terminal differentiation functions responsible for the sexually dimorphic adult cuticle (Figure 1; Ota et al., 1981).

The results presented here show that the sex determination regulatory hierarchy functions in the adult both to turn on and to maintain the synthesis of yolk proteins when they are being synthesized. In addition, these data show that control of YP gene expression by these regulatory genes occurs through the regulation of either YP gene transcription or YP transcript stability. Finally our results suggest that these regulatory genes control YP gene expression via their functioning within the fat body cells of the adult female where the YPs are synthesized.

Results

Influence of *tra-2^{ts}* on Yolk Protein Synthesis

There are two ways we could envision the *tra-2*⁺ function (and the sex determination regulatory hierarchy) acting to allow the synthesis of YPs in adult females but not males. One possibility is that at some time during development it acts in the progenitor cells of the adult fat body and ovarian follicular epithelium to determine irreversibly their sex and the sex of their descendants (i.e., their ability to syn-

thesize yolk proteins). Alternatively, *tra-2*⁺ function could be required in the adult at the time of YP synthesis to regulate the expression of these particular sexual differentiation functions. These possibilities can be distinguished by determining when the synthesis of YPs is temperature-sensitive in diplo-X flies homozygous for a temperature-sensitive allele of *tra-2*.

Chromosomal females homozygous for *tra-2^{ts2}* develop as phenotypic females when reared throughout development at the permissive temperature of 16°C, whereas they develop as males if reared at the restrictive temperature of 29°C (Belote and Baker, 1982). YP synthesis in individuals raised entirely at one temperature correlates with their cuticular sex. In *tra-2^{ts2}* homozygous adult females raised and kept at 16°C, YP synthesis occurs at a level comparable to that seen in the *tra-2^{ts2}/+* control females (Figures 2A and 2B). In XX; *tra-2^{ts2}* homozygotes raised and kept at 29°C there is essentially no YP synthesis detectable and the general synthetic profile of hemolymph proteins is nearly identical with that seen in XY control males (Figures 2C and 2D). These results confirm the findings of previous studies of null *tra-2* mutants, which showed that *tra-2*⁺ function is necessary for YP synthesis in females (Bownes and Nöthiger, 1981; Ota et al., 1981).

To study when wild-type *tra-2* function is needed to synthesize YPs, XX; *tra-2^{ts2}* homozygotes were reared at 29°C until they were 2 day old adults, shifted to 16°C for various periods of time, and assayed for YP synthesis. In these individuals synthesis of all three YPs is initiated within 24 hr following the shift to the permissive temperature (Figure 3, A–E). Thus, even in individuals that have followed the male developmental pathway throughout development, the initiation of *tra-2*⁺ activity in the adult is sufficient to turn on YP synthesis and, in at least this regard, to reverse their sexual commitment.

In wild-type flies YP synthesis is initiated near the time

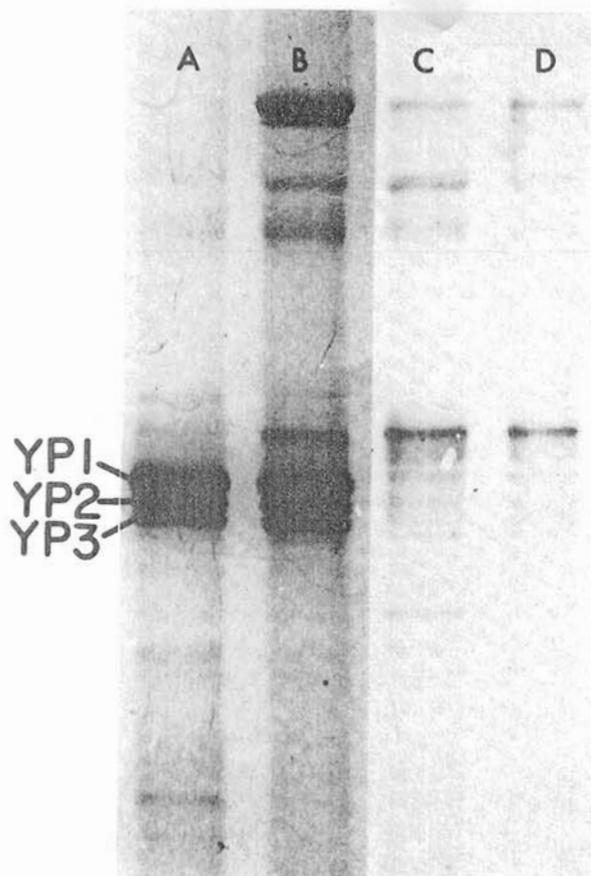


Figure 2. Hemolymph Yolk Protein Synthesis
Yolk protein synthesis was monitored by injecting adults of the indicated genotype and sexual phenotype with ^{35}S -methionine and, after 3 hr, separating the hemolymph proteins on a gradient SDS-polyacrylamide gel, followed by autoradiography. Lane A: XX; *tra-2^{ts2}*/ $+$ control females. Lane B: XX; *tra-2^{ts2}* females raised and kept at 16°C. Lane C: XY; *tra-2^{ts2}*/ $+$ control males. Lane D: XX; *tra-2^{ts2}* transformed females raised and kept at 29°C.

of eclosion in response to appropriate endocrine signals (Jowett and Postlethwait, 1980). To determine whether the initiation of YP synthesis by *tra-2** product is limited to a discrete phase (e.g., early adulthood), XX; *tra-2^{ts2}* homozygotes were reared to adulthood at 29°C and kept at that temperature for periods ranging from 2 to 14 days before being shifted to 16°C. Three days after the shift to the permissive temperature, YP synthesis was assayed. Levels of YP synthesis comparable to those observed in 2 day old wild-type females were initiated by a shift to the permissive temperature even after 12 days at 29°C (Figure 3, F–J). The strength of response in the 14 day sample was decreased but still detectable (not shown). These results demonstrate that *tra-2** maintains its regulation over the sexual state throughout much of adulthood, as do all other functions necessary for the initiation of YP synthesis.

To determine whether *tra-2** function was needed to maintain, as well as initiate, YP synthesis, XX; *tra-2^{ts2}* homozygotes were reared at the permissive temperature of 16°C until 2 days after eclosion, and then shifted to 29°C. YP synthesis was assayed after varying lengths of time at the restrictive temperature. In such shifted individ-

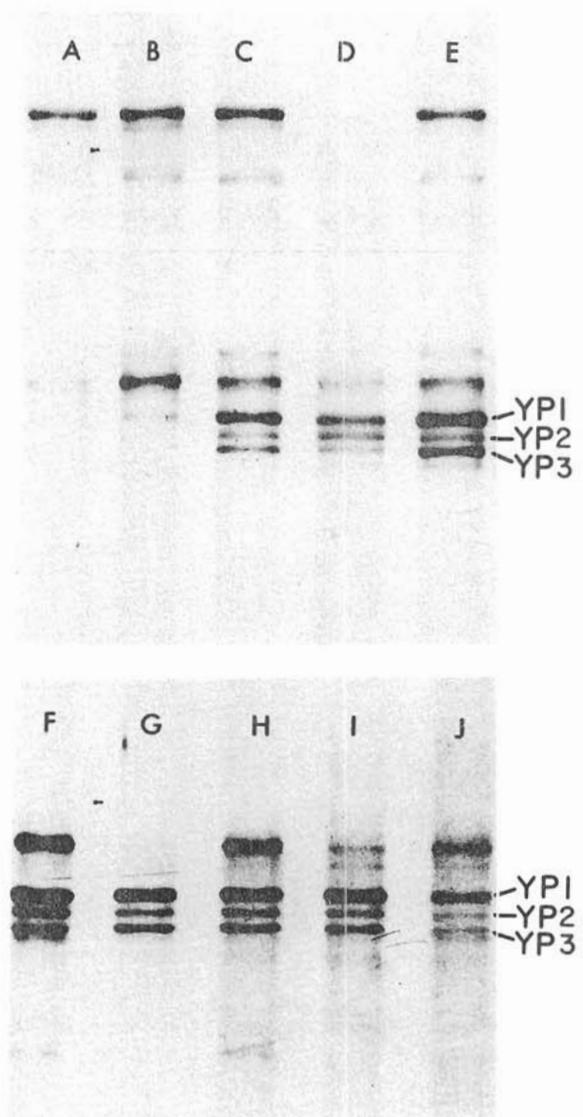


Figure 3. Induction of YP Synthesis in XX; *tra-2^{ts2}* Homozygotes following a Temperature Shift from 29°C to 16°C
Yolk protein synthesis in adults of indicated genotype and sexual phenotype was assayed as in Figure 2. Lane A: XX; *tra-2^{ts2}* transformed females raised and kept at 29°C. Lanes B–E: XX; *tra-2^{ts2}* transformed females raised and kept at 29°C until 2 days after eclosion and then shifted to 16°C for 2.5 hr (B), 21 hr (C), 3 days (D), or 4 days (E). Lane F: XX; *tra-2^{ts2}*/ $+$ control females. Lanes G–J: XX; *tra-2^{ts2}* transformed females shifted to 16°C for 3 days after having been raised and kept at 29°C until 2 days (G), 6 days (H), 9 days (I), or 12 days (J) after eclosion.

uals YP synthesis is maintained at a significant level for several days, but noticeably decreased by 3 days, and reached a very low level by 13 days at 29°C (Figure 4a). The most straightforward interpretation of these results is that *tra-2** function is required to maintain YP synthesis, but once YP synthesis is initiated removal of *tra-2** activity only gradually leads to a cessation of YP synthesis. Such a gradual attenuation of YP synthesis could, for example, be due to the presence of long-lived YP mRNAs. An alternative possibility is that once YP synthesis is initiated, *tra-2** function is not required for its maintenance and the

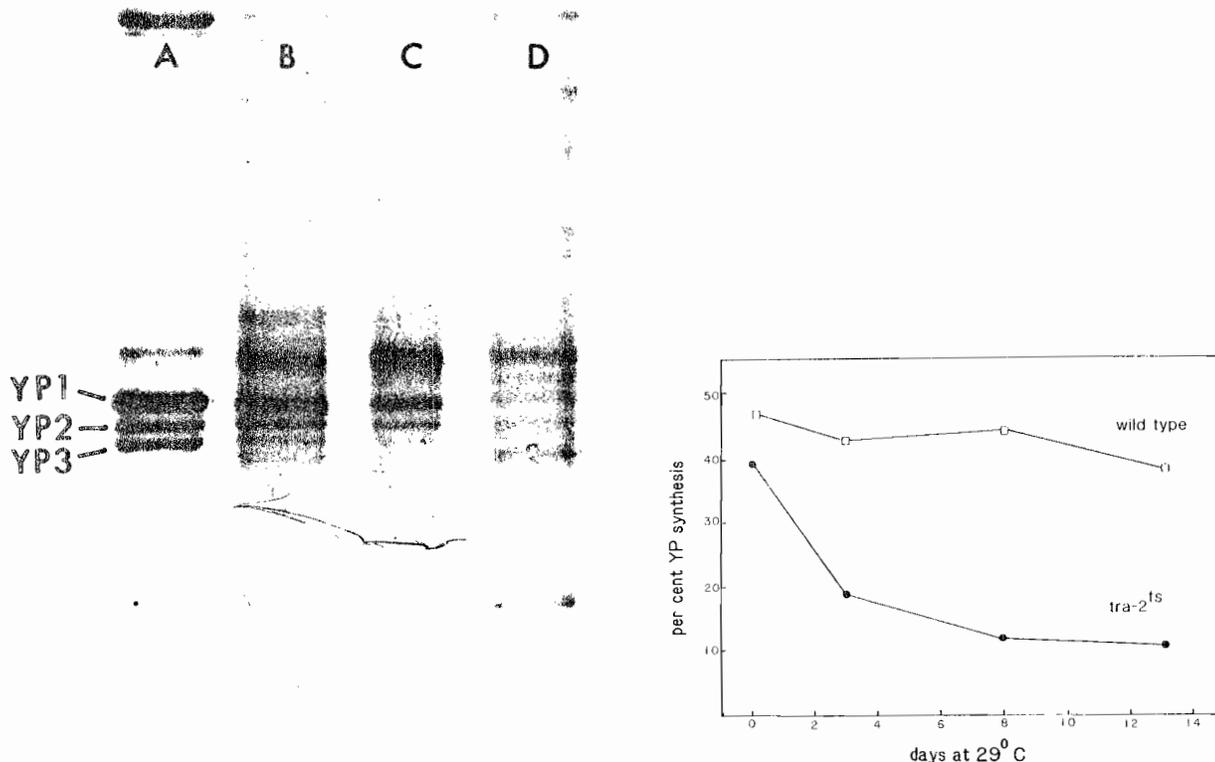


Figure 4. Turning off YP Synthesis in XX; *tra-2^{ts2}* Females following Temperature Shift to 29°C

(a) Yolk protein synthesis in adults of indicated genotype and sexual phenotype assayed as in Figure 2. Lane A: XX; *tra-2^{ts2}* females raised and kept at 16°C. Lanes B-D: XX; *tra-2^{ts2}* females raised and kept at 16°C until 2 days after eclosion and then shifted to 29°C for 2 days (B), 8 days (C), and 13 days (D).

(b) YP synthesis plotted as the percentage of total hemolymph protein synthesis in XX; *tra-2^{ts2}* females and wild-type control females. Flies were raised and kept at 16°C until 2 days after eclosion and then shifted to 29°C for the indicated times. Hemolymph proteins were labeled *in vivo* and separated on a gradient SDS-polyacrylamide gel as in Figure 2. Autoradiograms were scanned using a Kontes integrating densitometer.

decreasing rate of synthesis seen in this experiment is an artifact resulting from prolonged maintenance of flies at 29°C. This latter possibility does not appear to be the case: although YP synthesis in wild-type control females shifted to 29°C does decline as a function of time, it is still relatively high at 13 days following the shift, whereas YP synthesis is greatly reduced in XX; *tra-2^{ts2}* homozygous females by this time (Figure 4b). Together the above experiments show that *tra-2⁺* function is required in the adult female both to initiate and to maintain synthesis of yolk proteins.

Since YP synthesis is under endocrine control, one mechanism by which *tra-2⁺* could control YP synthesis is indirect, via regulation of anteriorly located endocrine tissues or the hormonal milieu (Handler and Postlethwait, 1978). Alternatively, *tra-2⁺* activity could be necessary within the cells of the fat body that synthesize YPs. To distinguish between these possibilities we examined abdomens of XX; *tra-2^{ts2}* homozygotes that have been separated from the anterior endocrine organs, to see if YP synthesis remains temperature-sensitive. Abdomens were isolated from XX; *tra-2^{ts2}* homozygotes reared at 29°C until 1 day after eclosion. These abdomens either were kept at 29°C or were shifted to 16°C for 24 to 36 hr. Groups of 12 abdomens were subsequently left untreated, or were treated with either 10⁻⁹ M 20-OH ecdysone or 10⁻³

M ZR-515, a juvenile hormone analog. Such hormone treatments have been shown sufficient to restore nearly normal levels of YP synthesis in isolated wild-type female abdomens (Handler and Postlethwait, 1978; Postlethwait and Handler, 1979). After an additional 12 hr incubation, abdomens were injected with ³⁵S-methionine and YP synthesis was analyzed (Figure 5). YPs were not produced in either untreated or hormonally treated abdomens kept at 29°C. After a shift to 16°C, untreated abdomens also failed to produce YPs, whereas shifted abdomens treated with either 20-OH ecdysone or juvenile hormone analog showed appreciable levels of YP synthesis. In these experiments synthesis of YP3 was noticeably lower than synthesis of YP1 and YP2. This difference is also seen in wild-type female abdomens that have been similarly treated with hormones (Postlethwait and Handler, 1979). The above results show that without *tra-2⁺* activity the presence of hormone by itself is insufficient to stimulate YP synthesis in the abdomen. This establishes that *tra-2⁺* activity is required in the abdomen, possibly within the cells of the fat body itself, for YP synthesis, and for modulation of this synthesis by hormones. This is consistent with the idea that *tra-2⁺* controls YP synthesis in a cell-autonomous manner, as it does all other previously examined aspects of sexual differentiation (Baker and Ridge, 1980; Wieschaus and Nöthiger, 1982).

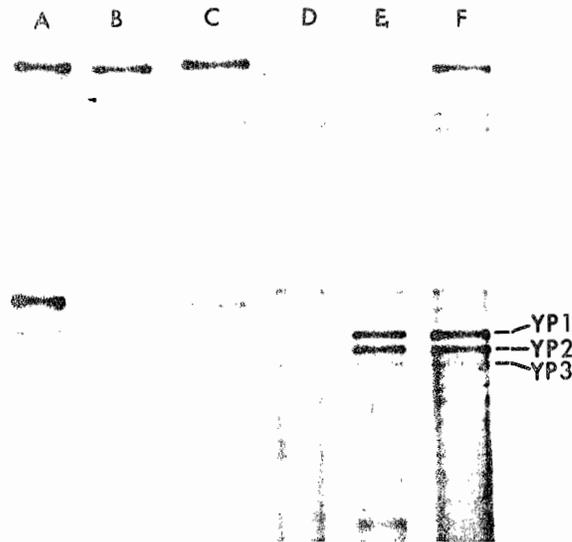


Figure 5. Yolk Protein Synthesis in Isolated Abdomens. Abdomens were isolated by ligation from 1 day old XX; *tra-2^{ts2}* adults that had been raised at 29°C. Abdomens were then either kept at 29°C (lanes A–C) or shifted to 16°C (lanes D–F) for 1 day before hormone treatment (see Experimental Procedures). YP synthesis was subsequently assayed as in Figure 2. Lanes A and D: no hormone treatment. Lanes B and E: abdomens treated with 20-hydroxyecdysone. Lanes C and F: abdomens treated with the juvenile hormone analog ZR-515.

Control of Yolk Protein RNA Levels

To determine whether the requirement of *tra-2* function for YP synthesis reflected regulation of the YP genes at the RNA level, the amounts of YP RNAs were measured following temperature shifts of XX; *tra-2^{ts2}* homozygotes. Flies were reared until 2 days after eclosion at either 16°C or 29°C and were then shifted to the other temperature for varying periods of time. Total RNA from whole flies was prepared for each time point and analyzed on Northern blots. The results closely parallel those obtained when YP synthesis is monitored (Figure 6). Females homozygous for *tra-2^{ts2}* kept continuously at 16°C have YP RNAs of the same sizes and amounts comparable to those of heterozygous female siblings. On the other hand, XX; *tra-2^{ts2}* homozygotes kept continuously at 29°C have little or no YP RNAs, as seen in wild-type males. This result is consistent with our observation that in chromosomal females homozygous for a non-ts allele of *tra-2*, YP RNA is not detected (data not shown). Following a shift from 29°C to 16°C an increase in YP3 RNA was detectable by 6 hr after the shift, and after 24 hr at 16°C reached levels seen in control females. Similar results were obtained for YP1 and YP2 except that the increase in RNA was not detectable until 24 hr following the shift to 16°C (Figure 6, A–I). When *tra-2^{ts2}* homozygous females were shifted from 16°C to 29°C the amount of YP RNA decreased gradually, analogous to what was seen when monitoring protein synthesis. After 1 day at the restrictive temperature the amount of YP RNA was comparable to the preshift levels, whereas by the third day amounts decreased substantially, reaching very low levels of YP RNA by 10 days at 29°C (Figure 6, lanes J–N).

These results indicate that the control of YP synthesis

by the *tra-2* gene occurs through the regulation of either YP gene transcription or YP transcript stability.

Cell-Type Specificity of *tra-2^{ts2}*-Induced YP Gene Expression

Previous organ culture studies showed that there are at least two sites of YP synthesis in adult females: in cells of the abdominal fat body and in some cells within the ovary (Gelti-Douka et al., 1974; Hames and Bownes, 1978; Postlethwait and Kaschnitz, 1978; Warren et al., 1979; Postlethwait et al., 1980). More recent experiments have shown that the follicular epithelium of vitellogenic oocytes is responsible for ovarian YP production (Brennan et al., 1982). YPs are also synthesized by cells in the thorax of adult females, presumably those of the thoracic fat body.

To ensure that the YP gene expression induced by temperature shifts of *tra-2^{ts}* homozygotes exhibited normal tissue specificity, ³H-labeled YP DNA probes were hybridized in situ to RNA in sections of XX; *tra-2^{ts2}* individuals. These flies had been reared to adulthood at 29°C and then either maintained at 29°C or shifted to 16°C 2 days after eclosion for periods of 1 or 3 days. As positive and negative controls for YP gene expression, a heterozygous female and a heterozygous male were sectioned with each experimental fly and hybridized with probe on the same microscope slide. Following autoradiography, the number of silver grains appearing over a given tissue provided a measure of the amount of RNA in those cells capable of hybridizing to the YP DNA probe.

Wild-type females exhibited a high level of silver grains over most, if not all, of the fat body cells in the abdomen, thorax, and head as well as the follicular epithelium of stage 10 follicles (Figure 7A). Adjacent sections of wild-type males had much lower levels of grains distributed uniformly over all tissues examined, providing a measure of nonspecific background labeling (Figure 7B). Consistent with the results of the RNA blot hybridizations described above, XX; *tra-2^{ts2}* homozygotes kept continuously at 29°C exhibited low levels of silver grains over all their tissues, as is seen in wild-type males (Figure 7C), confirming that these flies do not accumulate significant amounts of YP RNA. In contrast, sections of XX; *tra-2^{ts2}* homozygotes shifted as adults to 16°C for 1 or three days show substantial labeling over abdominal, thoracic, and head fat body cells, while other tissues remain labeled at the background level (Figure 7D). The degree of hybridization to fat body cells in the latter individuals is lower than that seen in wild-type females, suggesting that synthesis or accumulation of YP RNA is not as great as in normal females. Nonetheless, following hybridization with YP3 DNA probe the number of grains over the abdominal fat bodies of XX;*tra-2^{ts2}* flies shifted to 16°C was 4 to 6 fold greater than that seen in genetically identical flies kept at 29°C. Similar results were obtained when YP2 DNA was used as the hybridization probe (not shown).

The distribution of labeling in both wild-type and mutant females appeared to be uniform across all fat body cells in head, thorax, and abdomen. These results confirm previous findings that YP genes are transcribed in fat body cells of the abdomen and thorax as well as in the fol-



Figure 6. RNA Blot Hybridization of Nick-Translated YP1 DNA to Total RNA from Flies of the Indicated Genotype and Temperature Treatment

RNA samples are from flies of the following genotypes and sexual phenotypes. Lane A: XX; *tra-2^{ts2/+}* control females. Lane B: XX; *tra-2^{ts2}* females raised and kept at 16°C for 9 days. Lanes C–H: XX; *tra-2^{ts2}*-transformed females raised and kept at 29°C until 2 days after eclosion and were then shifted to 16°C for (C) 5 days, (D) 2 days, (E) 1 day, (F) 7.5 hr, (G) 5 hr, or (H) 2.5 hr. Lane I: XX; *tra-2^{ts2}* transformed females raised and kept at 29°C until 5 days after eclosion. Lanes J–N: XX; *tra-2^{ts2}* females raised and kept at 16°C until 2 days after eclosion and then shifted to 29°C for (J) 10 days, (K) 5 days, (L) 3 days, (M) 2 days, (N) 1 day. Lane O: XY; *tra-2^{ts2/+}* males at 29°C. The same filter hybridized to a probe containing an actin gene coding sequence (see Experimental Procedures) showed similar levels of hybridization across all lanes (not shown).

lular epithelium surrounding the oocyte (Brennan et al., 1982) and show, in addition, that they are expressed in the fat body of the head. Our observations also indicate that there are no conspicuous additional sites of YP RNA synthesis in shifted XX; *tra-2^{ts2}* homozygotes. Thus, with the exception of YP synthesis by stage 10 follicular epithelium, which is not present as a differentiated tissue in XX; *tra-2^{ts2}* homozygotes reared at 29°C, the tissue specificity of YP gene expression in down-shifted XX; *tra-2^{ts2}* individuals appears to be normal.

Discussion

Yolk protein genes of *Drosophila* are typical of many eukaryotic structural loci in that their expression is temporally and spatially regulated during development. In addition, they are regulated in a sex-specific manner. Studies on control of YP synthesis by the endocrine system and sex determination regulatory loci have identified a number of components responsible for the temporal and sexual regulation of YP gene expression. However, relatively little is known, as yet, about the regulatory processes acting during development to restrict YP gene expression to cells of the adult fat body and the ovarian follicular epithelium.

Here we have focused on the manner in which the regulatory hierarchy governing somatic sexual differentiation acts to produce the female-specific expression of the YP genes. The aim of these studies has been not only to elucidate this particular aspect of the control of YP gene expression but also to provide a paradigm of regulatory hierarchy control over expression of sexual differentiation functions.

Our experiments show that the function encoded by the *tra-2* locus is needed by the adult for YP synthesis at a time that coincides with YP gene expression. Moreover, wild-type *tra-2* function is needed both to initiate and to maintain synthesis of YPs. By shifting temperatures with *ts tra-2* mutants, YP synthesis can be induced at its normal time shortly after eclosion, and maintained for up to

at least 2 weeks. The products of the sex determination regulatory genes, as well as all other components necessary to control the initiation of YP synthesis, appear to be present or inducible throughout much of adulthood. These results strongly suggest that the sex determination regulatory hierarchy acts by controlling these three genes and, by inference, potentially all other genes encoding sex-specific differentiation functions at the time they are expressed rather than at an earlier stage of development. These results are consistent with previous findings on the control of cuticular differentiation by the *tra-2* locus, which showed that *tra-2* function was needed at different times, even within single cell lineages, for different aspects of sexual differentiation to occur (Belote and Baker, 1982).

The results described here also establish that *tra-2*⁺ controls the expression of YP genes and, by analogy, perhaps other loci encoding sexual differentiation functions by affecting YP RNA levels. Both Northern blot analysis and in situ hybridizations to RNA in tissue sections show that without *tra-2*⁺ function, YP transcripts are not accumulated, but following a restoration of *tra-2*⁺ function, YP transcripts are detectable within 24 hr. The effect of a removal of *tra-2*⁺ function on YP synthesis and YP RNA accumulation is less immediate: YP synthesis persists for up to 1 week after *tra-2^{ts2}* females are shifted to the restrictive temperature. Previous experiments indicated that the *tra-2^{ts2}* gene product probably becomes nonfunctional within 1 to 2 days at 29°C (Belote and Baker, 1982), suggesting that YP synthesis maintained after this time is caused by a persistent YP mRNA produced at 16°C. While this hypothesis is not exclusive, it has been suggested that YP mRNA is relatively stable based on the observation that YP synthesis occurs prodigiously in adult females without amplification of YP genes (Barnett et al., 1980). These results show that functional *tra-2* gene product is required both to initiate and to maintain the transcription (or increase the stability of transcripts) of these female-specific genes.

These data also show that cells of the adult fat body can change their sex (at least with respect to YP synthesis)

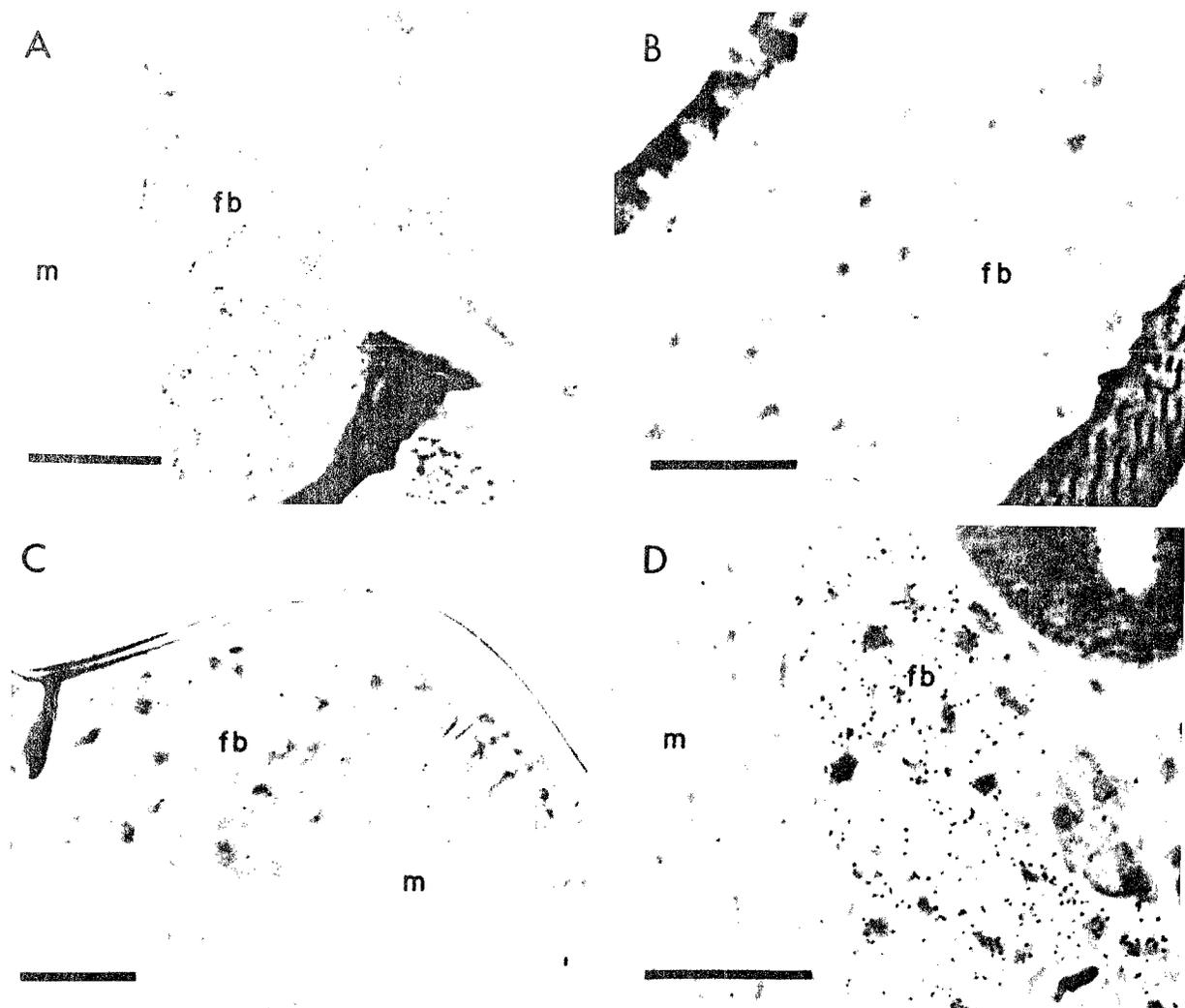


Figure 7. In situ Hybridization of ^3H -Labeled YP3 DNA Probe to RNA in Frozen Sections of Adult Flies

(A) XX; *tra-2^{ts2}/+* control female. (B) XY; *tra-2^{ts2}/+* control male. (C) XX; *tra-2^{ts2}* transformed female raised and kept at 29°C. (D) XX; *tra-2^{ts2}* transformed female raised and kept at 29°C until 2 days after eclosion and then shifted to 16°C for 3 days. fb = fat body. m = muscle. Bar = 25 μm .

quite readily, without extensive rounds of cell division. Adult fat body cells, like those of most somatic tissues, do not appear to undergo division in the adult (Rizki, 1978), although some DNA synthesis in these cells (which may be polyploid) is detected by ^3H -thymidine incorporation (J. M. Belote and B. S. Baker, unpublished data). Moreover, XX; *tra-2^{ts2}* individuals are capable of initiating YP synthesis when shifted to 16°C, even after extensive γ -irradiation, which prevents cell division (A. M. Handler, unpublished data). YP synthesis results from a change in the sexual state of fat body cells and not from some other tissue, as fat body cells are the major, if not the only, site of YP gene expression in *tra-2^{ts2}* individuals induced by temperature shift to synthesize YPs.

Previous work suggested that the *tra-2* locus does not act directly to control genes encoding terminal sexual differentiation functions but, rather, does so indirectly by controlling the expression of another regulatory gene in this hierarchy: the *doublesex* (*dsx*) locus (Baker and Ridge, 1980; Belote and Baker, 1982). Genetic analysis

has suggested that the *dsx* locus is bifunctional and acts in a negative manner to control sexual differentiation (Figure 1). In males (1X:2A) *dsx⁺* functions to repress female differentiation; in females (2X:2A), *dsx⁺* represses male differentiation. The *tra-2⁺* and *transformer* (*tra⁺*) gene products are thought to act in chromosomally female individuals to maintain the *dsx⁺* locus in the female mode of expression. In chromosomal males the *tra⁺* and *tra-2⁺* loci are not expressed; as a consequence *dsx⁺* is expressed in the male mode.

That *tra-2⁺* functions to control YP synthesis via its regulation of the *dsx⁺* locus is consistent with the finding that, when YP synthesis is used as a diagnostic phenotype for sex, a pattern of epistatic interactions between sex determination mutants is observed to be identical with the one that led to the above model (Ota et al., 1981). These data are consistent with the hypothesis that active control of YP gene expression occurs in males in which the expression of *dsx⁺* in the male mode leads to repression of YP synthesis. In females the *tra-2⁺* function acts to switch *dsx⁺* from

the male mode to the female mode of expression thereby removing the repression of YP synthesis. Thus, functional *tra-2* product is needed to switch *dsx** from a male-specific to a female-specific expression mode thereby initiating YP synthesis in XX; *tra-2^{ts}* individuals that had developed as males. Similarly, continuation of YP synthesis requires a continuation of functional *tra-2** product to prevent expression of *dsx** in the male mode, which would shut off YP synthesis. The *dsx** locus has been cloned (B. S. Baker and M. F. Wolfner, unpublished data) and the available data on its expression are consistent with this model. In particular, transcripts homologous to *dsx** are found in adult males (B. S. Baker, unpublished data).

As is evident from what we know about the regulation of YP genes, a structural gene concerned with a particular aspect of sexual differentiation must have information not only about the sex of the cell it is in, but also about the cell type and developmental stage, in order to be properly expressed. Thus, at some level information from regulatory hierarchies controlling different aspects of development must be communicated to each gene encoding a differentiation function. The regulatory loci, such as *tra-2*, that control sexual differentiation share many formal genetic properties with the homeotic genes that regulate other aspects of Drosophila development (García-Bellido, 1977; W. K. Baker, 1978; Lewis, 1978; Baker and Ridge, 1980; Belote and Baker, 1982). The result reported here that *tra-2** function is needed both to turn on and to maintain expression of the YP genes may reflect how sex determination regulatory genes and possibly other homeotic loci, function to regulate the genes under their control. If so, it suggests that hierarchies controlling various aspects of development interact to regulate the expression of differentiation functions at the level of the loci, which themselves encode final differentiation functions. Under this hypothesis each particular structural gene encoding a terminal differentiation function would require a specific combination of signals from these regulatory hierarchies to render it expressible.

Regulation of the expression of YP (vitellogenin) genes has also been studied in oviparous vertebrates, notably frogs (Ryffel et al., 1977; Westley, 1979; Wahli, et al., 1981) and chickens (Lazier, 1978; Elbrecht et al., 1981; Burch and Weintraub, 1983). In both species the phenomenon is quite analogous to that of Drosophila since vitellogenin genes are normally expressed only in female liver and are controlled by the steroid hormone estrogen (Ryffel et al., 1977; Lazier, 1978). However, the vertebrates and Drosophila differ significantly in how they achieve sex-specific vitellogenin synthesis. During vertebrate development the vitellogenin genes in the liver cells of both sexes become committed to expression in the adult, but only in females do estrogen and estrogen-receptor levels reach the concentrations needed to induce expression (Lazier, 1978; Westley, 1979). Thus the sex-specific control of vitellogenin synthesis in vertebrates is achieved by modulating the functioning of the endocrine system. In Drosophila this does not appear to be the case. Titers of hormone (20-hydroxyecdysone) are not appreciably different between males and females at any time during postlarval develop-

ment (Handler, 1982). Moreover, only limited YP synthesis can be stimulated in males by exogenously supplied 20-hydroxyecdysone and even then only with exceptionally high levels of this hormone (Bownes, 1982; Postlethwait et al., 1980). YP synthesis in males cannot be stimulated at all with juvenile hormone (Postlethwait et al., 1980; Shirk et al., 1983). Thus, Drosophila does not appear to bring about the sex-specific regulation of YP synthesis by modulating hormone levels. Instead, in Drosophila the sex specificity of YP gene expression appears to be brought about by the sex determination regulatory hierarchy acting in the fat body of males to render the YP genes unable to express regardless of hormone induction, whereas in females the YP genes are not repressed and can thus respond to hormone.

Experimental Procedures

Drosophila Cultures and Mutant Phenotypes

Drosophila melanogaster were grown at 16°C or 29°C on cornmeal-molasses-yeast-agar medium containing propionic acid and supplemented with live yeast. Adults were collected within 12 hr after eclosion, classified according to phenotype, and stored at the temperature of culture for 2 days. They were then transferred to fresh food and either kept at that same temperature or shifted to the other temperature until used.

The *tra-2^{ts2}* mutation was induced by ethyl methanesulfonate (Belote and Baker, 1982). XX; *tra-2^{ts2}* flies raised continuously at 29°C develop as phenotypic males with rudimentary gonads. Such flies are indistinguishable from chromosomal females that have been transformed into males by a null *tra-2* mutation. Flies of the same genotype raised at 16°C develop as morphologically normal females that are marginally fertile. XY; *tra-2^{ts2}* individuals are morphologically normal males that are sterile when raised at 29°C but fertile when raised at 16°C (Belote and Baker, 1983).

The chromosome bearing the *tra-2^{ts2}* mutation was marked with mutation *brown (bw)* and balanced over the *In(2LR) CyO, dp^{h1} Cy pr cn²* chromosome. Chromosomal males (XY) carried the dominant eye shape marker *Bar of Stone (B^S)* as a small duplication on the Y chromosome. XX; *tra-2^{ts2}* homozygotes could thus be unambiguously identified independently of their sexual phenotype as non-*Bar^S*, non-Cy, brown-eyed flies. Descriptions of other genetic markers used can be found in Lindsley and Grell (1968).

Yolk Protein Synthesis

Animals were injected with 1 μ Ci ³⁵S-methionine (1100 Ci/mmol; Amersham) dissolved in Drosophila Ringers. They were then incubated at the appropriate temperature for 3 hr. Hemolymph was extracted by injecting their abdomens with approximately 0.3 μ l Ringers solution and then puncturing the abdomen and withdrawing as much fluid as possible with a drawn-out microcapillary. The hemolymph was then diluted in 0.4 ml distilled water and proteins precipitated with trichloroacetic acid (Bensadoun and Weinstein, 1976). The acetone-washed precipitate was dissolved in 40 μ l of sample buffer (Hames and Bownes, 1978) and run on a 9%–12% sodium dodecyl sulfate polyacrylamide gradient slab gel prepared according to O'Farrell (1975). After electrophoresis, the gel was stained with Coomassie blue, dried down on filter paper, and autoradiographed for periods ranging from 12 to 48 hr. For some experiments gels were prepared for fluorography with Enhance solution (New England Nuclear) and film exposed at -70°C. For some samples, YPs were quantified by scanning densitometry of autoradiograms using a Kontes integrating densitometer following the one-dimensional separation of hemolymph proteins.

YP Synthesis in Isolated Abdomens

Abdomens were ligated by tying nylon monofilament thread between the thorax and first abdominal segment, whereupon the thorax and head were cut away with iridectomy scissors. Isolated abdomens were maintained at 29° or 16°C in humid chambers to prevent desiccation. For hormone treatments abdomens were either injected with approxi-

mately 0.2 μ l of 10^{-5} M 20-OH ecdysone (Rohto Pharm.) dissolved in 10% ethanol-Ringers solution, or received topical application of 10^{-3} M juvenile hormone analog (ZR-515, a gift from Zoecon Corp.) dissolved in acetone. Twelve hours after hormone treatment, synthesis of hemolymph YP was assayed as described for intact animals.

RNA Extraction and Analysis

For each time point, 20–30 adult flies were frozen in liquid nitrogen and stored at -70°C until extracted. Frozen flies were extracted by homogenization in 2 ml of 0.1 M Tris (pH 8.9), 0.1 M NaCl, 30 mM EDTA, 1% sarcosyl, 0.4% diethylpyrocarbonate, 1.5 ml phenol, and 1.5 ml chloroform: isoamyl alcohol (24:1). The resulting aqueous phase was made 0.1 M in sodium acetate (pH 5), and ethanol-precipitated at -20°C . RNA was pelleted at $17,300 \times g$ for 10 min, washed once with 70% ethanol, dried under vacuum, and resuspended in 100 μ l sterile H_2O . Yields were approximately 250 μ g of RNA.

For Northern transfers, 66 μ g RNA was denatured in formaldehyde as described by Mehdy et al., (1983). Sixty micrograms of this RNA was run on a formaldehyde/1.4% agarose gel, transferred to nitrocellulose, and baked (Thomas, 1980). The remaining 6 μ g was run on a parallel gel and stained with ethidium bromide to monitor the quality and quantity of the RNA.

Probes for hybridization were synthesized from DNA fragments corresponding to the YP1, YP2, or YP3, or to actin coding sequences (to confirm comparable loadings of each RNA sample). The YP genes cloned in pBR322 (Barnett et al., 1980) were kindly provided by Dr. P. Wensink (Brandeis Univ.), and actin probes were from a cDNA clone, aDm108D11 (Wolfer, Ph.D. Thesis, Stanford, Univ. 1980). *Drosophila* sequences were excised from the vector using appropriate restriction enzymes. The fragments were separated by electrophoresis on 7% agarose gels and purified using DE81 paper (Dretzen et al., 1981). DNA fragments were labeled with ^{32}P by nick translation (Rigby et al., 1977; Maniatis et al., 1976) and hybridized to the filters overnight at 42°C in 50% formamide, $5 \times$ SSPE, 0.25 mg/ml denatured salmon sperm DNA, 10% w/v sodium dextran sulfate (Wahl et al., 1979), 0.1% SDS, and 0.02% each polyvinylpyrrolidone, Ficoll, bovine serum albumin. Filters were washed in $0.1 \times$ SSPE, 0.1% SDS for 3 hr at 50°C and autoradiographed at -70°C using an intensifying screen and Kodak XAR5 film.

For reuse of the filters, hybridized counts were removed by a 2–3 hr incubation at 80°C in 0.1% SDS, 1 mM EDTA. Filters were autoradiographed to check for residual radioactivity.

In Situ Hybridization to RNA in Tissue Sections

The hybridization of labeled DNA probes to RNA in tissue sections followed the procedure of Akam (1983). A wild-type control female, a shifted or unshifted XX:*tra-2^{ts2}* fly, and a wild-type control male were etherized, placed side by side in a drop of OCT embedding compound, and quick-frozen with CO_2 . Ten to twelve micron-thick sections were cut such that all three flies were concomitantly sectioned. The sections were fixed, dehydrated, stored, and treated just prior to hybridization as described (Akam 1983).

Cloned YP DNAs (Barnett et al., 1980) were ^3H -labeled by nick translation using ^3H -dCTP, ^3H -dGTP, and ^3H -dTTP (New England Nuclear; specific activity 25.0, 9.5, and 40.0 Ci/mmol, respectively) as radiolabeled nucleotides in the reaction. Probes having specific activities in the range of $1-2 \times 10^7$ cpm/ μ g were obtained. These probes were digested with pancreatic DNAase I to yield molecules that, when denatured, average about 100–200 nucleotides in length as determined by polyacrylamide gel electrophoresis.

Tritiated DNA (2×10^4 cpm/ μ l) was resuspended in hybridization solution, denatured, applied to slides, and hybridized (for 24 hr) as described by Akam (1983). Coverslips were then floated off in posthybridization wash buffer consisting of 50% formamide, 600 mM NaCl, 1 mM EDTA, 10 mM Tris-Cl, pH 7.5, and slides were washed repeatedly in this buffer (eight changes over 18 hr) at 25°C . Sections were then dehydrated by passing the slides once through 70% ethanol/300 mM ammonium acetate, pH 7.0, for 5 min and twice through 90% ethanol/300 mM ammonium acetate, pH 7.0, for 5 min. After air drying, slides were dipped in Kodak NTB2 nuclear track emulsion, and autoradiographed at 4°C for 2–5 weeks. After developing the autoradiograms, the tissue was stained with Azure B and coverslips affixed with Permount.

Acknowledgments

We would like to thank Dr. Pieter Wensink for providing us with the YP gene clones. This work was supported by grants from U.S. Public Health Services (GM 23345, GM 07199 and HD16519) and the National Science Foundation (PCM-8202812). M. F. W. and K. J. L. were supported by Damon Runyan-Walter Winchell Cancer Fund Fellowships (DRG 436 and DRG 351).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 9, 1984; revised December 3, 1984

References

- Akam, M. E. (1983). The location of *Ultrabithorax* transcripts in *Drosophila* tissue sections. *EMBO J.* 2, 2075–2084.
- Baker, B. S., and Belote, J. M. (1983). Sex determination and dosage compensation in *Drosophila melanogaster*. *Ann. Rev. Genet.* 17, 345–397.
- Baker, B. S., and Ridge, K. (1980). Sex and the single cell: on the action of major loci affecting sex determination in *Drosophila melanogaster*. *Genetics* 94, 383–423.
- Baker, W. K. (1978). A genetic framework for *Drosophila* development. *Ann. Rev. Genet.* 12, 451–470.
- Barnett, T., and Wensink, P. C. (1981). Transcription and translation of yolk protein mRNA in the fat bodies of *Drosophila*. In *ICN-UCLA Symposium. Developmental Biology Using Purified Genes*, D. D. Brown and C. F. Fox, eds. (New York: Academic Press) Vol. 23, pp. 97–106.
- Barnett, T., Pachl, C., Gergen, J. P., and Wensink, P. C. (1980). The isolation and characterization of *Drosophila* yolk protein genes. *Cell* 21, 729–738.
- Belote, J. M., and Baker, B. S. (1982). Sex determination in *Drosophila melanogaster*: analysis of *transformer-2*, a sex transforming locus. *Proc. Natl. Acad. Sci. USA* 79, 1568–1572.
- Belote, J. M., and Baker, B. S. (1983). The dual functions of a sex determination gene in *Drosophila melanogaster*. *Dev. Biol.* 95, 512–517.
- Bensadoun, A., and Weinstein, D. (1976). Assay of proteins in the presence of interfering materials. *Anal. Biochem.* 70, 241–250.
- Bownes, M. (1982). The role of 20-hydroxyecdysone in yolk polypeptide synthesis by male and female fat bodies of *Drosophila melanogaster*. *J. Insect Physiol.* 28, 317–328.
- Bownes, M., and Nöthiger, R. (1981). Sex determining genes and vitellogenin synthesis in *Drosophila melanogaster*. *Mol. Gen. Genet.* 182, 222–228.
- Brennan, M. D., Weiner, A. J., Goralski, T. J., and Mahowald, A. P. (1982). The follicle cells are a major site of vitellogenin synthesis in *Drosophila melanogaster*. *Dev. Biol.* 89, 225–236.
- Burch, J. B. E., and Weintraub, H. (1983). Temporal order of chromatin structural changes associated with activation of the major chicken vitellogenin gene. *Cell* 33, 65–76.
- Cline, T. W. (1979). A male-specific mutation in *Drosophila melanogaster* that transforms sex. *Dev. Biol.* 72, 266–275.
- Cline, T. W. (1983). The interaction between daughterless and sex-lethal in triploids: a novel sex-transforming maternal effect linking sex determination and dosage compensation in *Drosophila melanogaster*. *Dev. Biol.* 95, 260–274.
- Cline, T. W. (1984). Autoregulatory functioning of a *Drosophila* gene product that establishes and maintains the sexually determined state. *Genetics* 107, 231–277.
- Dretzen, G., Bellard, M., Sassone-Corsi, P., and Chambon, P. (1981). A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. *Anal. Biochem.* 112, 295–298.
- Elbrecht, A., Williams, D. L., Blue, M. L., and Lazier, C. B. (1981). Differential ontogeny of estrogen responsiveness in the chick embryo liver. *Can. J. Biochem.* 59, 606–612.
- García-Bellido, A. (1977). Homeotic and atavistic mutations in insects.

- Am. Zool. 17, 613-629.
- Gelti-Douka, H., Gingeras, T. R., and Kambysellis, M. P. (1974). Yolk proteins in *Drosophila*: identification and site of synthesis. J. Exp. Zool. 187, 167-172.
- Hames, B. D., and Bownes, M. (1978). Synthesis of yolk proteins in *Drosophila melanogaster*. Insect Biochem. 8, 319-328.
- Handler, A. M. (1982). Ecdysteroid titers during pupal and adult development in *Drosophila melanogaster*. Dev. Biol. 93, 73-82.
- Handler, A. M., and Postlethwait, J. H. (1977). Endocrine control of vitellogenesis in *Drosophila melanogaster*: effects of the brain and corpus allatum. J. Exp. Zool. 201, 389-403.
- Handler, A. M., and Postlethwait, J. H. (1978). Regulation of vitellogenin synthesis in *Drosophila* by ecdysterone and juvenile hormone. J. Exp. Zool. 206, 247-254.
- Hovemann, B., Galler, R., Walldorf, V., Kupper, H., and Bautz, E. K. (1981). Vitellogenin in *Drosophila melanogaster*: sequence of the yolk protein 1 gene and its flanking regions. Nucl. Acids Res. 9, 4721-4734.
- Hung, M. C., Barnett, T., Woolford, C., and Wensink, P. C. (1982). Transcript maps of *Drosophila* yolk protein genes. J. Mol. Biol. 154, 581-602.
- Jowett, T., and Postlethwait, J. H. (1980). Regulation of yolk polypeptide synthesis in *Drosophila* ovaries and fat body by 20-hydroxyecdysone and a juvenile hormone analog. Dev. Biol. 80, 225-234.
- Lazier, C. (1978). Ontogeny of vitellogenic response to estradiol and of the soluble nuclear estrogen receptor in embryonic chicken liver. Biochem. J. 174, 143.
- Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. Nature 276, 565-570.
- Lindsley, D. L., and Grell, E. H. (1968). Genetic variations of *Drosophila melanogaster*. Carnegie Inst. Wash. Publ. 627.
- Lucchesi, J. C., and Skripsky, T. (1981). The link between dosage compensation and sex differentiation in *Drosophila melanogaster*. Chromosoma 82, 217-227.
- Mahowald, A. P. (1972). Ultrastructural observations on oogenesis in *Drosophila*. J. Morphol. 137, 29-48.
- Maniatis, T., Kee, S. G., Efstratiadis, A., and Kafatos, F. C. (1976). Amplification and characterization of a β -globin gene synthesized in vitro. Cell 8, 163-182.
- Maroni, G., and Plaut, W. (1973). Dosage compensation in *Drosophila melanogaster* triploids. I. Autoradiographic study. Chromosoma 40, 361-377.
- Mehdy, M. C., Ratner, D., and Firtel, R. A. (1983). Induction and modulation of cell-type-specific gene expression in Dictyostelium. Cell 32, 763-771.
- O'Farrell, P. H. (1975). High resolution two dimensional resolution of proteins. J. Biol. Chem. 250, 4007-4021.
- Ota, T., Fukunaga, A., Kawabe, M., and Oishi, K. (1981). Interactions between sex transformation mutants of *Drosophila melanogaster*. I. Hemolymph vitellogenins and gonad morphology. Genetics 99, 429-441.
- Postlethwait, J. H., and Handler, A. M. (1979). The roles of juvenile hormone and 20-hydroxyecdysone during vitellogenesis in isolated abdomens of *Drosophila melanogaster*. J. Insect Physiol. 25, 455-460.
- Postlethwait, J. H., and Jowett, T. (1981). Regulation of vitellogenesis in *Drosophila*. In Regulation of Insect Development and Behavior, F. Sehna, A. Zabza, J. J. Menn, and B. Cymborowski, eds. (Wroclaw: Wroclaw Technical Univ. Press) vol. 2, pp. 591-628.
- Postlethwait, J. H., and Kaschnitz, R. (1978). The synthesis of *Drosophila melanogaster* vitellogenins in vivo, in culture, and in a cell-free translation system. FEBS Lett. 95, 247-251.
- Postlethwait, J. H., Bownes, M., and Jowett, T. (1980). Sexual phenotype and vitellogenins in *Drosophila*. Dev. Biol. 79, 1205-1216.
- Rigby, P. W. J., Dieckmann, M., Rhodes, C., and Berg, P. (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237-251.
- Rizki, T. M. (1978). Fat body. In The Genetics and Biology of *Drosophila*, 2B. M. Ashburner, and T. R. F. Wright, eds. (London: Academic Press) pp. 561-601.
- Ryffel, G. U., Wahli, W., and Weber, R. (1977). Quantitation of vitellogenin messenger RNA in the liver of male *Xenopus* toads during primary and secondary stimulation by estrogen. Cell 11, 213-221.
- Shirk, P. D., Minoo, P., and Postlethwait, J. H. (1983). 20-Hydroxyecdysone stimulates the accumulation of translatable yolk polypeptide gene transcript in adult male *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 80, 186-190.
- Thomas, P. S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77, 5201-5205.
- Van Deusen, E. B. (1976). Sex determination in germline chimeras of *Drosophila melanogaster*. J. Embryol. Exp. Morphol. 37, 173-185.
- Wahl, G. M., Stern, M., and Stark, G. R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. USA 76, 3683-3687.
- Wahli, W., Dawid, I. B., Ryffel, G. U., and Weber, R. (1981). Vitellogenesis and the vitellogenin gene family. Science 212, 298-304.
- Warren, T. G., Brennan, M. D., and Mahowald, A. P. (1979). Two processing steps in the maturation of vitellogenin polypeptide in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 76, 2848-2852.
- Westley, B. (1979). The relationship of the estrogen receptor to the induction of vitellogenin in chicken and *Xenopus* liver. Differentiation 15, 67-72.
- Wieschaus, E., and Nöthiger, R. (1982). The role of the transformer genes in the development of the genitalia and analia of *Drosophila melanogaster*. Dev. Biol. 90, 320-334.