MOLECULAR AND CELLULAR BIOLOGY, Oct. 1986, p. 3388–3396
0270-7306/86/103388-09$02.00/0

Vol. 6, No. 10

5′-Flanking Sequence Required for Regulated Expression of a Muscle-Specific *Drosophila melanogaster* Actin Gene

PAMELA K. GEYER AND ERIC A. FYRBERG*

Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

Received 17 March 1986/Accepted 6 June 1986

We have functionally tested derivatives of a muscle-specific *Drosophila melanogaster* actin gene in which 5′-flanking sequences have been deleted or rearranged. From our results we conclude that approximately 1,000 nucleotides of 5′-flanking sequence are required for wild-type levels of mRNA accumulation during flight muscle development. Derivatives having 875 or 865 nucleotides of upstream sequence could be expressed normally, but were prone to influence by flanking foreign DNA sequences. Derivatives retaining 600 or fewer nucleotides of flanking DNA did not direct detectable levels of mRNA accumulation. The sequence residing between −919 and −640 could be inverted and yet retain normal function. Deletion of this sequence reduced mRNA accumulation markedly, but did not affect its spatial localization, suggesting that elements which confer tissue specificity reside close to the point of transcription initiation.

More precise understanding of actin gene regulation will enhance our comprehension of many aspects of cellular development and function. Actin is found in all eucaryotic cells and is a major component of the cytoskeleton. In addition, actin polymers form the backbone of thin filaments in muscle. It has been established that in most metazoans, several isotypic variants of actin (isoforms) are specified by a closely related family of genes, members of which are differentially expressed both temporally and spatially (see, for example, Fyrberg et al., [6]). However, mechanisms implementing tissue-specific actin mRNA accumulation are poorly understood, as are those which coordinate actin gene expression with that of other contractile protein genes.

Tissue- and temporal-specific gene expression results in part from the interaction of regulatory factors with specific sequences located within or immediately flanking the gene of interest (7). Therefore, we have begun to identify *cis*-linked regulatory regions required for the transcription of particular actin genes of the fruit fly, *Drosophila melanogaster*. Our strategy is to alter previously cloned genes by in vitro mutagenesis and to subsequently reintroduce such derivatives into germ line chromosomes by p-element-mediated transformation (23). The temporal- and tissue-specific pattern in which transduced genes are expressed can be examined in any number of transformed lines. Generally the p-element-mediated approach constitutes a rigorous functional test for identifying control regions because the transduced gene is present in low copy number in all cells and experiences the entire developmental program of the animal.

One of the *Drosophila* actin genes, *act88F*, lends itself well to analyses of regulatory mechanisms. The primary advantage is that this particular gene is expressed only within indirect flight muscles. Since these fibers are required only for flight, mutant alleles do not result in lethality. Recently, several mutants harboring deleterious *act88F* alleles have been characterized, and their phenotypic abnormalities in the flight muscles have been described (8, 12, 14). One of these mutants, *raised*, proved to be an ideal recipient strain for p-element-mediated *act88F* transformation. In this mutant the "resident" *act88F* gene directs accumulation of only 10 to 20% of wild-type mRNA levels, a deficiency which leads to severe disruption of flight muscle myofibrils. Previously we have shown that both the mRNA deficiency and myofibrillar abnormalities can be corrected by introducing a wild-type copy of the *act88F* actin gene which includes only 4.5 kilobases (kb) of 5′ flanking sequence (14).

In this work we have extended our initial *raised* transformation experiment by constructing and testing a series of *act88F* derivatives having deleted or altered 5′ flanking sequences. From our results we conclude that approximately 1,000 nucleotides of 5′-flanking sequence are required for wild-type mRNA accumulation. Furthermore, our data suggest that multiple *cis*-linked elements are required for appropriate *act88F* transcription. We discuss these findings in light of data obtained with other eucaryotic genes.

MATERIALS AND METHODS

Plasmid construction. The *Xba*I-*Sac*I fragment of the *act88F* actin gene was subcloned into a derivative of the Carnegie 4 vector (19), in which the *Eco*RI site of the polylinker has been replaced by one recognized by *Sal*I. The actin gene-containing fragment includes 1,420 nucleotides of 5′-flanking sequence and approximately 400 nucleotides of 3′-flanking sequences. Deletions of this plasmid (D-1420 Carnegie 4 Sal) were constructed by Bal 31 nuclease digestion, repaired with the Klenow fragment of DNA polymerase, and ligated to kinased *Sal*I linkers with T4 DNA ligase. The sequence from −1420 to +78, as well as breakpoints of all deletions, are illustrated in Fig. 8.

Plasmid XhoDel was constructed by deleting the 5′ *Xho* fragment from D-1420 Carnegie 4 Sal, which includes nucleotides −919 to −640 (see Fig. 8). Plasmid XhoInv contains an inversion of the same *Xho* fragment. All reconstructions were subcloned into pCarnegie 20 (19) so that the 5′ end of the actin gene was adjacent to the p-element and the 3′ end was adjacent to the xanthine dehydrogenase (*rosy*) gene.

Germ line transformation. Germ line transformation was carried out essentially as described by Spradling and Rubin (23). The recipient *Drosophila* strain was the double mutant *rosy raised* (ry rsd). DNA concentrations used for injection were 300 μg of the Carnegie 20 (actin gene-containing) construct per ml and 100 μg of the "wings-clipped" helper plasmid pπ 25.7wc (11) per ml. Larvae surviving the injec-

* Corresponding author.

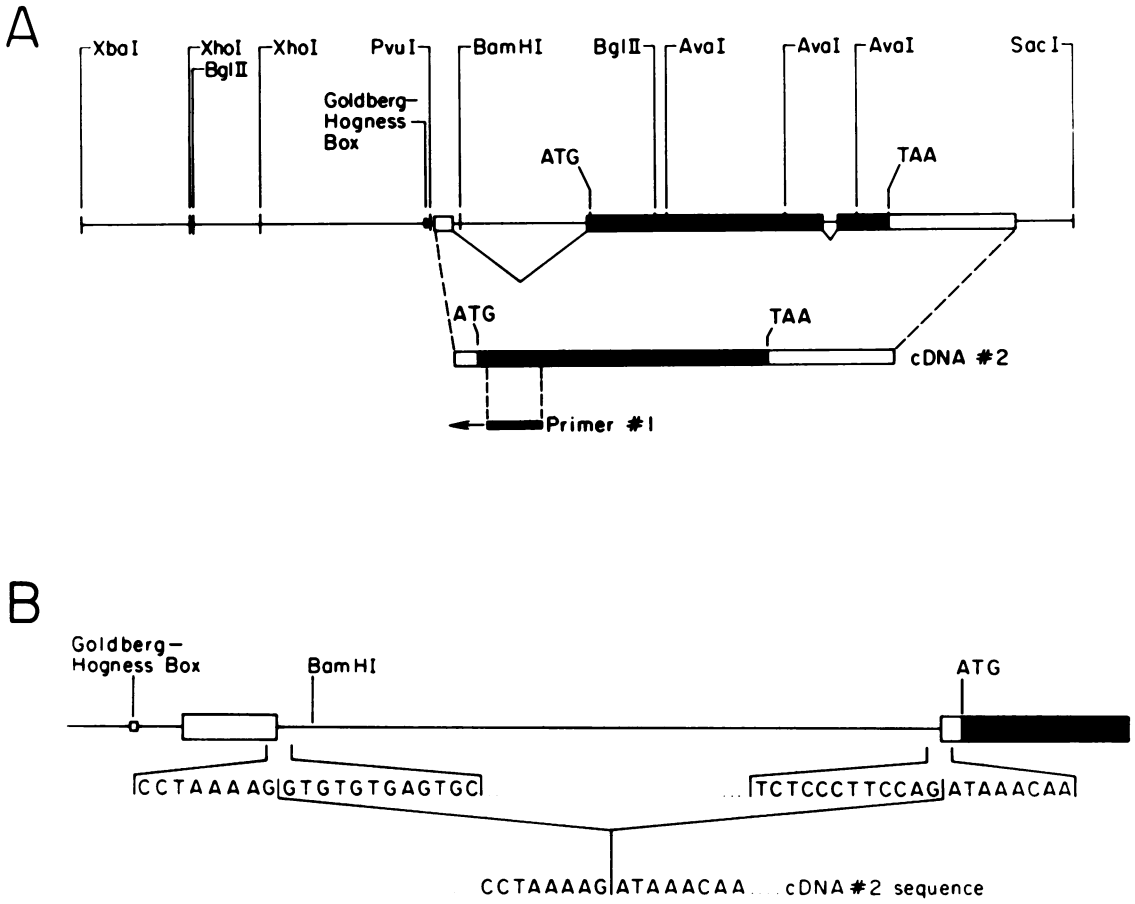

FIG. 1. Organization of the *act88F* actin gene. (A) Arrangement of a 3.9-kb *Xba*I-*Sac*I fragment containing the *act88F* actin gene. Solid blocks represent the protein-coding region, and open blocks represent sequences that encode the 5′ and 3′ untranslated regions of the mRNA. The gene contains two introns, one in the 5′ untranslated region and one within codon 307 of the protein-coding region. The lower portion illustrates the structure of a nearly full-length cDNA clone recovered from the adult stage λgt10 library of B. Yedvobnick, and also the primer (a *Taq*I fragment containing codons 10 through 71) used to synthesize a 5′ extended probe. (B) Sequences of the first intron junctions, as determined by comparisons of genomic and cDNA clones.

tions were transferred to standard food vials and allowed to develop to adults. These adults (G0) were backcrossed to *ry rsd* flies. Individual ry^+ G1 flies from a single G0 adult were mated to *ry rsd* flies, and the progeny of a cross which gave a 1:1 segregation of the *ry* phenotype were used to establish homozygous lines. Southern blot analysis of genomic DNA was used to confirm the number of integrations in each established line. Usually 100 embryos were injected. Of these, normally 50% survived the injection procedure. The number of transformed flies obtained ranged between 30 and 40% of the fertile adults.

RNA preparation and analysis. RNA was isolated from synchronously developing larvae, pupae, or newly eclosed adults by the sodium dodecyl sulfate (SDS)-phenol method (22). Larvae and adults were homogenized directly in SDS buffer, whereas pupae were first frozen on dry ice, ground into a fine powder, and then transferred to SDS buffer. Homogenates were extracted several times with phenol-Sevag (Sevag is a 24:1 mixture of chloroform-isoamyl alcohol). Nucleic acids were precipitated with ethanol and polyadenylated [poly(A)$^+$] RNA selected by chromatography on oligo(dT)-cellulose (1).

Northern analysis was performed essentially as described by Fyrberg et al. (6). Poly(A)$^+$ RNA (5 μg per lane) was electrophoresed on 1.5% agarose gels containing 2.2 M formaldehyde. RNA was transferred to nitrocellulose and hybridized to a mixture of two ^{32}P-labeled probes. The first detects *hsp83* gene transcripts (clone 301.1 of Holmgren et al. [10]) and was used to standardize the amount of RNA per gel lane. The second was a probe which specifically hybridizes to transcripts of the *act88F* actin gene (act88F, probe B; Fyrberg et al. [6]). Filters were prehybridized at 50°C for 12 to 20 h in 50% (vol/vol) formamide–0.8 M NaCl–0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–0.01% Sarkosyl–5× Denhardt solution–1 mg of denatured calf thymus DNA per ml. They were then hybridized to 10^6 cpm of nick-translated probes (specific activity $>5 \times 10^7$ cpm/μg) per ml at 50°C in the buffer above with 10% (wt/vol) dextran sulfate added and 500 μg of denatured calf thymus DNA per ml rather than 1 mg/ml. Hybridizations were done for 40 to 48 h with gentle agitation. After hybridization, the filters were washed once at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% Sarkosyl–0.02% sodium pyrophosphate and then several times at 50 or 55°C in 0.2× SSC–0.05% Sarkosyl–0.01% sodium pyrophosphate or at 75°C in 2× SSC–0.1% sodium pyrophosphate–0.1% SDS, and then exposed to Kodak XAR-5 X-ray film with Du Pont Lightning-Plus intensifying

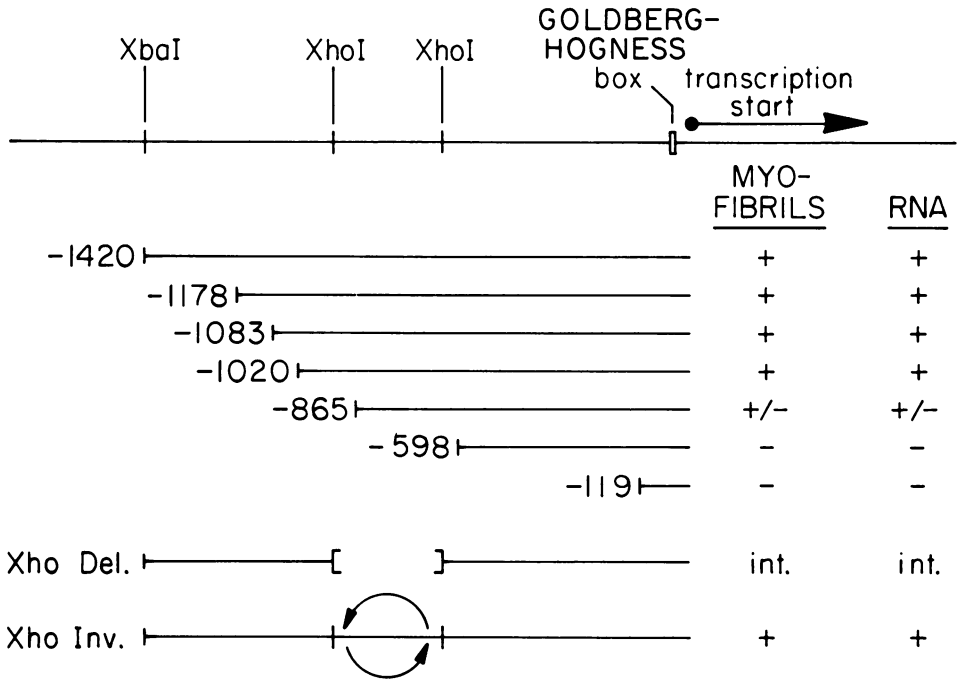

FIG. 2. Deleted and rearranged transposons used for germ line transformation. The top line is a restriction map of the 5′ end of the *act88F* actin gene, with the positions of the Goldberg-Hogness box and transcription start point illustrated. Below it is the series of actin "alleles" used for germ line transformation. Each construct has the same 3′ end (corresponding to the *Sac*I site shown in Fig. 1), and all were subcloned in the same orientation into the *Sal*I site of the Carnegie 20 vector. The transposons are named by the number of nucleotides remaining upstream of the cap site, D-1420, D-1178, etc. The resulting phenotype engendered in flight muscles of *ry rsd* double mutants by each transposon is indicated to the right of each gene. int. signifies that levels of mRNA accumulation were intermediate between wild type and that characteristic of the *ry rsd* strain.

screens. A graded series of exposures was prepared, and the amount of hybridization was quantitated by scanning autoradiographs with a densitometer.

Determination of myofibril morphology. Myofibrils from the indirect flight muscle of newly eclosed adults were dissected in Ringer solution (5) containing 0.2% Triton X-100 and visualized by phase microscopy.

DNA sequence analysis. Fragments to be sequenced were labeled with ^{32}P-labeled nucleotides at their 3′ termini with the Klenow fragment of DNA polymerase. Labeled fragments were sequenced by the procedure of Maxam and Gilbert (16).

RESULTS

Mapping the *act88F* transcript. As the first step in our analysis we defined the 5′ border of the *act88F* transcript. In initial experiments, a *Taq*I fragment including codons 10 to 71 of the protein-coding region was hybridized to late pupal poly(A)$^+$ mRNA and extended by using reverse transcriptase and ^{32}P-labeled nucleotides. Hybridization of this probe to Southern blots of restriction-digested *act88F* DNA suggested that a small 5′ exon resided between the *Pvu*I and *Bam*HI sites (Fig. 1A). To substantiate this notion we isolated a nearly full-length cDNA from an adult-stage λgt10 library (kindly provided by Barry Yedvobnick). Comparison of genomic and cDNA sequences confirmed that a 552-nucleotide intron interrupted the 5′ untranslated region (Fig. 1B). Twenty-eight nucleotides upstream from the 5′ border of the cDNA we found a suitable eucaryotic mRNA capping sequence (CCATTTGT), and 27 nucleotides further upstream lay a suitable Goldberg-Hogness box (ATATAAA). Recent data (16b) are in agreement with our results and strongly suggest that the A of the capping sequence is the first transcribed nucleotide. Previously published work of Sanchèz et al. (20) failed to delineate the correct transcription initiation point because the mRNA used in the mapping experiments was isolated during developmental stages when the *act88F* gene is not expressed.

Our analyses of the 3′ end of the *act88F* gene demonstrated that transcription did not proceed past the *Sac*I site shown in Fig. 1. Blot hybridization experiments revealed that our cDNA clone contained only sequences to the left of the *Sac*I site (data not shown). Furthermore, oligo(dT)-primed cDNA representing late pupal mRNA hybridized only upstream of the same site.

Analyses of actin gene expression in transformed lines. To identify *cis*-linked regulatory elements of *act88F*, we examined how sequential deletion of 5′-flanking DNA affected the functioning of the gene during flight muscle development. *act88F* derivatives containing various amounts of 5′-flanking sequences were inserted into pCarnegie 20, a p-element-derived vector containing a functional copy of the xanthine dehydrogenase (XDH) (rosy) gene. These plasmids were used to transform the doubly mutant *rosy raised* (*ry rsd*) strain. *ry* flies have abnormal eye color, due to low XDH activity, and the mutation is rescuable by transformation with the wild-type gene encoding the enzyme. In all cases we used eye color rescue as the primary marker for transformed individuals. In lines established from such transformants, we subsequently examined whether the *act88F* derivative linked to the XDH gene was capable of correcting flight muscle defects associated with the *rsd* mutation. Since myofibril

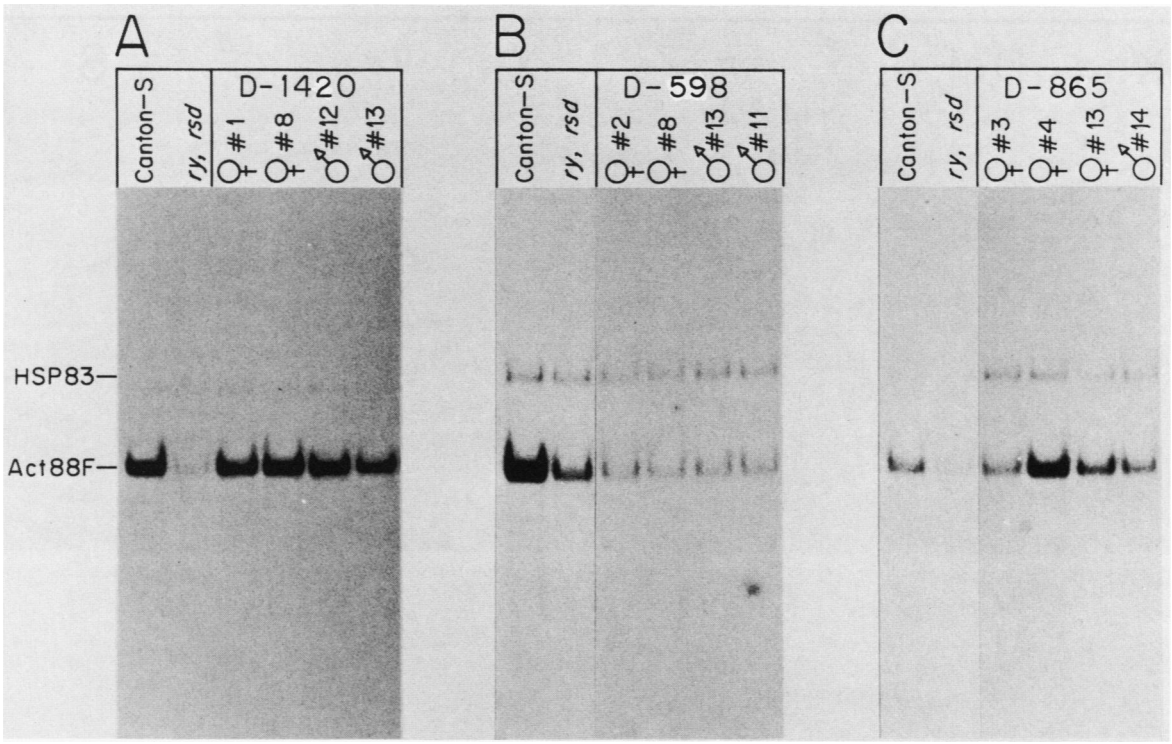

FIG. 3. Northern blot analysis of deleted *act88F* transposons. RNA blots of several lines harboring either D-1420, D-598, or D-865 transposons. In each case approximately 5 μg of poly(A)$^+$ RNA was electrophoresed on a 1.5% agarose–formaldehyde gel and transferred to nitrocellulose. Blots were hybridized simultaneously with an *act88F* gene-specific probe and an *hsp83* probe, which served as an internal control for the amount of RNA loaded. D-1420 functioned as well as the resident wild-type (Canton-S) allele. D-598, on the other hand, did not function detectably. Lines transformed with this construct accumulated no more *act88F* mRNA than did the *ry rsd* host. D-865 transposons functioned in a position-dependent manner. Some lines harboring this transposon accumulated as much or more mRNA than the Canton-S strain, while others accumulated only slightly more than the *ry rsd* host strain.

assembly ultimately depends on *act88F* mRNA accumulation, we reasoned that this assay would reflect transcriptional activity of deleted genes. To ensure that the functioning of a particular *act88F* construct was not unduly influenced by the site of chromosomal integration, several transformed lines were examined for each deletion.

Both the structure and function of our series of deletions are summarized in Fig. 2. Lines transformed with D-1420, D-1178, D-1083, and D-1020 derivatives had myofibrils indistinguishable from those of the wild-type (Canton-S) flies. Lines transformed with D-598 and D-119 did not have myofibrils and were indistinguishable from *rsd* homozygotes. Transformants containing D-865 (or D-875) had a range of phenotypes. In some lines the myofibrils were normal, whereas in other lines the myofibrils were totally absent.

To obtain a more accurate reflection of the transcriptional activity of these deleted genes, the accumulation of the *act88F* mRNA was examined. Poly(A)$^+$ RNA was isolated from *Canton-S*, *ry rsd*, and homozygous transformed flies during late pupation, the time of maximal accumulation of *act88F* mRNA (6). RNA was electrophoresed on 1.5% agarose–formaldehyde gels, transferred to nitrocellulose, and simultaneously hybridized with a ^{32}P-labeled *act88F* gene-specific probe and a ^{32}P-labeled heat shock protein (hsp) 83 clone. The *hsp83* mRNA level is relatively invariant during *Drosophila* development (15) and is unaffected by the *rsd* mutation (14). We therefore used it as an internal control for the amount of RNA loaded per lane. Levels of *act88F* and *hsp83* mRNAs were determined by densitometric scanning of several different autoradiographic exposures of at least two independent gels. These results are also summarized in Fig. 2, with more quantitative data (accumulation of *act88F* mRNA relative to that observed in Canton-S) summarized in Table 1.

As judged by their ability to direct mRNA accumulation, the series of deletion derivatives again fell into three classes. Northern blots representing each are shown in Fig. 3. The first class (Fig. 3A), comprising the four constructs having more than 1,000 nucleotides of flanking sequences (D-1420, D-1178, D-1083, and D-1020), accumulated wild-type levels of *act88F* mRNA. Furthermore, stocks having two homozygous copies of these derivatives accumulated twice as much *act88F* mRNA as Canton-S, directly demonstrating that RNA accumulation is proportional to gene dosage (Table 1). From these results we conclude that sequences upstream of −1020 are not essential for transcription of *act88F*. The second class (Fig. 3C) consisted of derivatives having 865 or 875 nucleotides of flanking DNA. These constructs were extremely sensitive to chromosome context. In some lines *act88F* mRNA accumulation was equal to or greater than that seen in Canton-S flies, while other lines accumulated mRNA at levels somewhat less than the wild type. Either these constructs lack particular regulatory sequences or they have insufficient flanking DNA to buffer regulatory sequences from *cis*-acting effects originating within surrounding chromosomal DNA. The third class of constructs comprised those having less than 598 nucleotides of 5′-flanking DNA (Fig. 3B). These constructs did not direct detectable mRNA accumulation. All lines carrying such derivatives

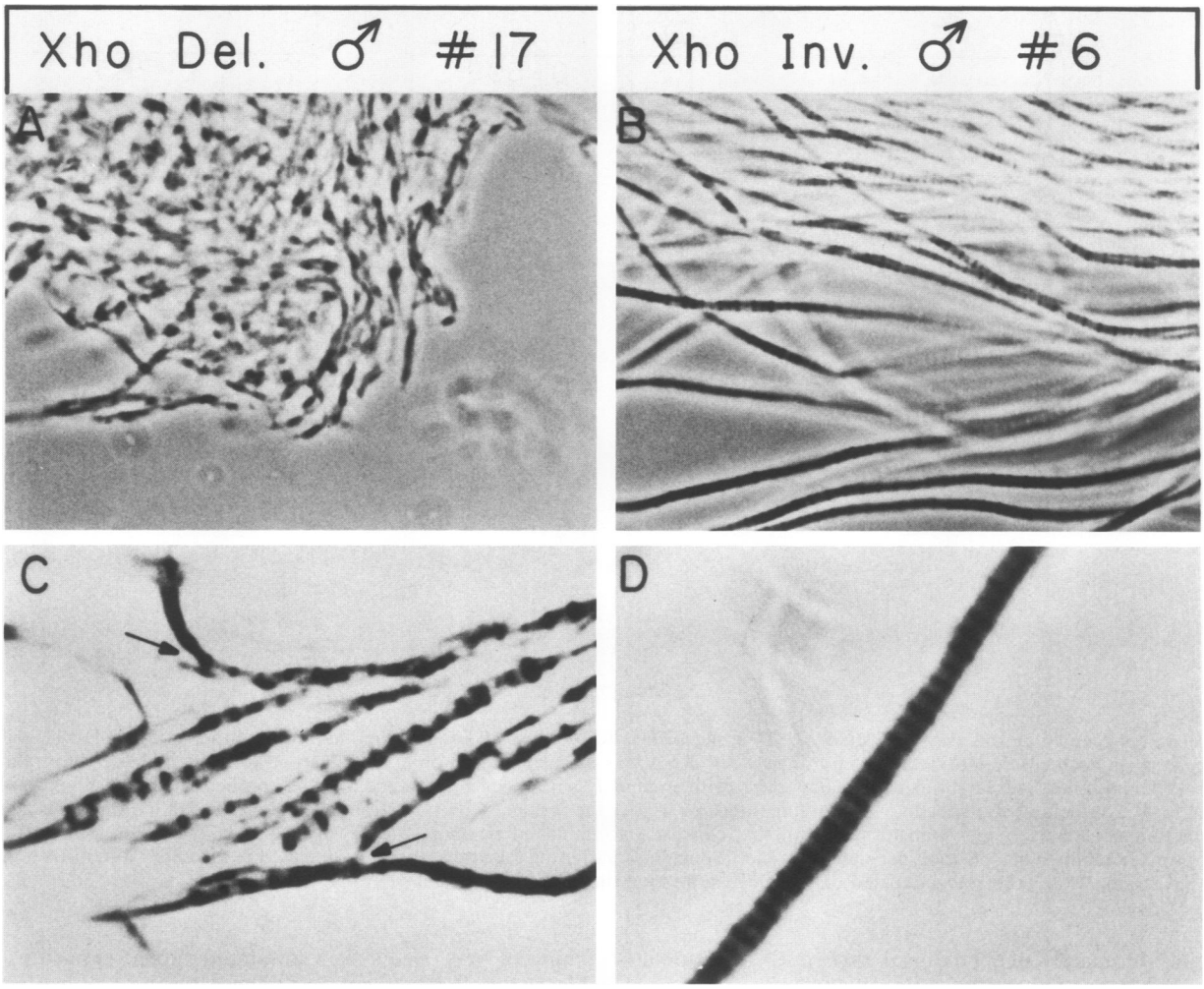

FIG. 4. Phase micrographs of flight muscle myofibrils in lines harboring XhoDel and XhoInv transposons. Myofibrils of recently eclosed adults were dissected in Ringer solution and viewed by phase microscopy. Flight muscles from all XhoDel lines (A and C) were grossly abnormal. The dissected myofibrils were short and frequently twisted or bent. At higher magnification they can be seen to be torn or bifurcated (arrows, panel C). In contrast, the flight muscles of XhoInv lines (B and D) could not be differentiated from wild type. The myofibrils were straight and had precisely ordered sarcomeres.

accumulated *act88F* mRNA at the same level as the host *ry rsd* strain.

Bidirectionally acting essential function of upstream sequences. To more rigorously establish that sequences residing between −900 and −600 are required for proper expression of the *act88F* actin gene, we performed additional experiments. In the first we deleted only these sequences from the D-1420 construct. Digestion of D-1420 with *Xho*I, followed by religation, eliminated only the sequences residing between −919 and −640. A transposon having this internal deletion (referred to as XhoDel) was introduced into the *ry rsd* stock, and both myofibril morphology and mRNA accumulation were examined (Fig. 4 and 5). As can be seen by inspection of Fig. 4A and C, myofibrils of the XhoDel-transformants were very abnormal. While arranged as sarcomeres, they were extremely weak in the structural sense and nearly always broken or torn during dissection. These structural abnormalities suggest that the XhoDel derivative is incapable of directing wild-type levels of transcription. Quantitation of steady-state mRNA levels confirmed that this was the case. As can be seen in Fig. 5 (and also by reference to Table 1), XhoDel transformants consistently underproduced *act88F* mRNA by two- to threefold, demonstrating that the deleted region contains essential sequences.

We next examined whether sequences located between the *Xho*I sites could function in an orientation-independent manner. Although the *Xho*I fragment is required for accumulation of wild-type mRNA levels, it was considerably removed from the point at which mRNA transcription is initiated. Furthermore, deletion of the *Xho*I fragment did not abolish mRNA production or alter mRNA size, suggesting that the promoter region was unaffected. Both of the aforementioned observations indicate that the element located within the *Xho*I fragment is analogous to viral or cellular enhancer sequences (2, 3). To determine whether the *Xho*I fragment could function in the reverse orientation, we constructed a D-1420 derivative in which the *Xho*I fragment was inverted and assessed its ability to rescue *rsd* muscle defects. This construct directed nearly normal myofibril formation, a result which we confirmed in three different lines (Fig. 4). In all three of these lines we observed wild-type levels of mRNA accumulation (Fig. 5, Table 1). Collectively, these results demonstrate that sequences within the

TABLE 1. Relative steady-state levels of *act88F* mRNA[a]

D. melanogaster strain	Sex	Fly no.	No. of transposons per diploid genome	Relative mRNA accumulation[b]
ry rsd			None	0.19
D-1420	♀	1	2	0.70
	♀	8	2	1.20
	♂	12	2	0.92
	♂	13	2	0.88
D-1178	♂	4	1	0.55
	♂	6	4	1.80
D-1083	♀	5	2	1.05
	♀	6	2	.99
	♂	11	4	2.00
D-1020	♀	5	2	1.50
D-875	♂	2	2	1.00
	♂	4	2	0.41
	♀	18	2	1.00
D-865	♀	3	2	0.23
	♀	4	2	1.37
	♀	13	2	1.40
	♂	14	4	0.56
D-598	♀	2	2	0.13
	♀	8	2	0.12
	♂	11	2	0.10
	♂	13	2	0.07
D-119	♀	4	4	0.09
	♀	5	4	0.10
	♀	6	2	0.10
	♂	10	4	0.24
XHODel	♂	4	2	0.41
	♂	8	2	0.56
	♀	16	2	0.51
	♀	17	2	0.35
XHOInv	♂	4	4	1.65
	♀	6	2	1.00
	♂	10	2	0.96

[a] Steady-state *act88F* mRNA levels were established by quantitative RNA blot hybridization, as outlined in Materials and Methods. All values represent the average derived from two or three different pupal-stage mRNA preparations. In the case of XhoInv ♂ no. 10, we examined three mRNA preparations, one of which (0.66, illustrated in Fig. 5) was rejected because it was inconsistent with both myofibril morphology and the remaining two readings, which were averaged to arrive at the 0.96 value.

[b] Relative to Canton-S strain (1.00).

*Xho*I fragment are capable of functioning in either orientation.

Effects of deletions and rearrangements on specificity of *act88F* expression. Lastly we examined whether temporal- and tissue-specific expression of *act88F* was affected in the D-865 and XhoDel constructs. In both of these "alleles," steady-state mRNA levels differed considerably from those observed with the wild-type *act88F* gene, and we therefore wondered whether the spatial pattern of expression was similarly altered. Distribution of actin mRNA was examined in newly eclosed adults in the following fashion. Total RNA was isolated from either the thoraces (T) or pooled heads, legs, and abdomens (H+L+A) of 10 flies. As a standard for RNA levels, total RNA isolated from 10 undissected flies was loaded in parallel lanes. RNA samples were fractionated on an agarose gel, transferred to nitrocellulose, and hybridized with a mixure of ^{32}P-labeled *hsp83* and *act88F* probes. The results of these experiments (Fig. 6) demonstrated that essentially all the *act88F* RNA was localized within the thoraces. Thus, the tissue specificity of *act88F* expression was not detectably altered in either construct. Similar experiments were performed in which *act88F* mRNA levels were quantitated during various developmental stages of particular transformed lines. These results (Fig. 7) demonstrated that the pattern of *act88F* mRNA accumulation in D-865 was identical to that seen in D-1020. In both cases, *act88F* mRNA was detectable only in mid-stage to late-stage pupae and recently eclosed adults, as is the case in normal *Drosophila* development. Additional experiments performed in our laboratory revealed that XhoDel directed an identical pattern of mRNA accumulation (data not shown). Collectively, these experiments suggest that the regulatory element located between the *Xho*I sites is not essential for spatially and temporally regulated expression.

DISCUSSION

In this manuscript we report our initial investigation of *cis*-linked sequences which regulate the expression of a muscle-specific actin gene. Having precisely defined the transcribed region, we deleted and rearranged upstream sequences and tested the in vivo functioning of such derivatives. The ability of each such allele to direct both myofibril formation and mRNA accumulation was examined after introduction into germ line chromosomes of the *act88F* hypomorph *rsd*. Sequential 5′ deletion experiments demonstrated that not more than 1,020 nucleotides of 5′-flanking sequence are required for regulated expression and that at least 865 nucleotides are needed to direct any detectable mRNA accumulation. Other experiments reported here confirmed that sequences residing between −919 and −640 are

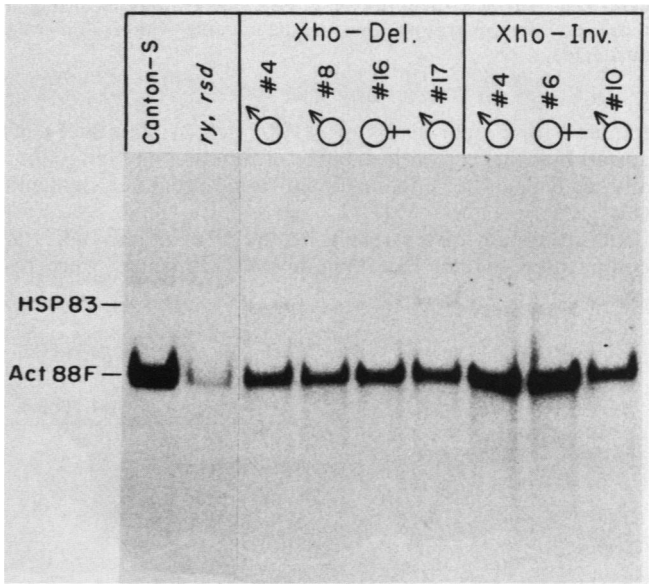

FIG. 5. Northern blot analysis of lines carrying XhoDel and XhoInv transposons. All four of the XhoDel lines accumulated significantly more *act88F* mRNA than the *ry rsd* host strain, but none accumulated as much as the wild-type stock. This observation is compatible with the abnormal myofibrillar phenotype in this genotype. On the other hand, two of the XhoInv lines (♂ no. 4 and ♀ no. 6) accumulated as much mRNA as wild-type flies on a per-gene basis (see Table 1). Again, this result is compatible with the myofibrillar phenotype, which in this case was completely normal. The third XhoInv line (♂ no. 10) appeared, from the blot illustrated, to accumulate reduced levels of *act88F* mRNA, a result incompatible with its normal myofibril morphology. This discrepancy was almost certainly due to improper developmental staging of pupae from which this particular mRNA preparation was isolated. Other mRNA samples from the same line revealed that it accumulated as much *act88F* mRNA as the wild-type strain (Table 1). Note that the *hsp83* bands in the control lanes are not aligned with those of the experimental samples.

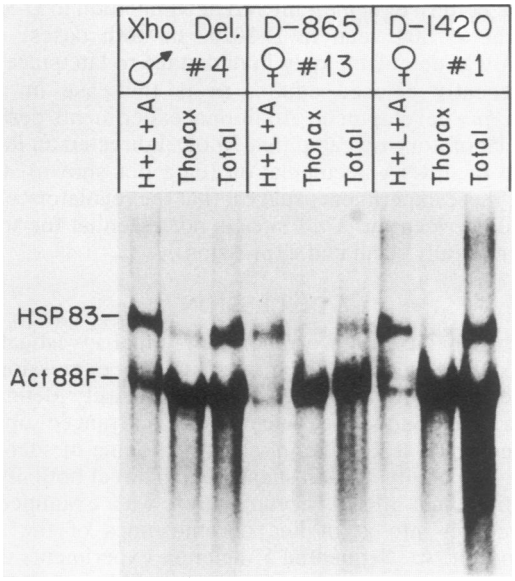

FIG. 6. Spatial distribution of *act88F* mRNA in transformed lines. The tissue distribution of *act88F* mRNA in particular transformed lines is illustrated. Total RNA was isolated from 10 recently eclosed adults, from 10 thoraces, or from pooled heads, legs, and abdomens (H+L+A) of 10 adults. After electrophoresis and blotting, RNA was hybridized to *act88F* and *hsp83* probes as previously described. The vast majority of *act88F* mRNA was localized in thoraces, demonstrating that tissue-specific expression was unaffected.

required for proper levels of *act88F* transcription and suggested that this region is capable of functioning bidirectionally, as is usual for enhancers and some promoter elements (17).

Interpretation of the results of the 5′ deletion studies is complicated by the fact that deleted sequences were replaced by others that were not necessarily neutral. All of our transposons were constructed so that following integration into the genome, the 5′ end of the deletion derivative was separated from genomic sequence only by the 550-nucleotide p-element terminus. This particular p-element sequence has never been shown to influence gene activity dramatically, but flanking genomic DNA might be expected to influence transcription and thus our conclusions. To control for this aspect of our experiments, we took two precautions. First, we selected transformants based on the activity of the rosy gene rather than on *act88F* expression and thus minimized selecting a biased collection. Second, we analyzed the function of each construct in several transformed lines, each bearing the transposed sequence in a distinct chromosomal location. The majority of our constructs functioned consistently in the different lines, suggesting that nearby genomic sequences do not have a large influence.

The function of two of our constructs (D-875 and D-865) did fluctuate widely in different lines, however. Since these two constructs had the least flanking DNA of any construct that directed RNA accumulation, the observation suggests that either they are inadequately buffered from flanking genomic DNA or a portion of a regulatory region has been removed, rendering the gene more prone to influence by flanking DNA. Similar position effects have been noted in other studies (4, 13, 18, 24), and in all cases they are most pronounced in functional derivatives having the least flanking DNA. In the case of the white locus, Levis et al. (13) have provided reasonable evidence that derivatives which are most prone to position effects lack distal regulatory elements. However, further elucidation of this phenomenon will require defining the regions actually bound by regulatory proteins, using the technique of DNA footprinting.

Taken with the results of the 5′ series of deletions, the impaired function of the XhoDel derivative indicates the involvement of multiple regulatory elements in *act88F* transcription. Derivatives having 865 or 875 nucleotides of flanking sequence were strongly position dependent, while

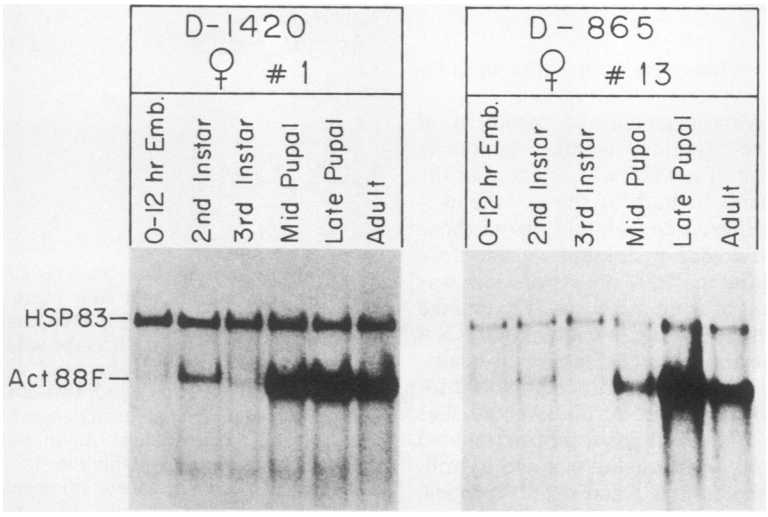

FIG. 7. Temporal pattern of mRNA accumulation in transformed lines. The left panel illustrates mRNA accumulation in a line transformed with the D-1420 construct. mRNA accumulated only in mid- to late-stage pupae and in newly eclosed adults. Hybridizing actin mRNA seen in second instar larvae was the product of the *act57A* (larval muscle-specific) gene. This transcript was detected due to its weak homology with the *act88F* probe (which contains 121 nucleotides of the protein-coding region). D-865 transcripts accumulated with the same pattern (right panel), demonstrating that the temporal pattern was not altered by removal of upstream sequence. The pattern observed in lines transformed with the XhoDel construct was also identical (data not shown), demonstrating that sequences residing between −919 and −640 do not determine the temporal pattern of accumulation.

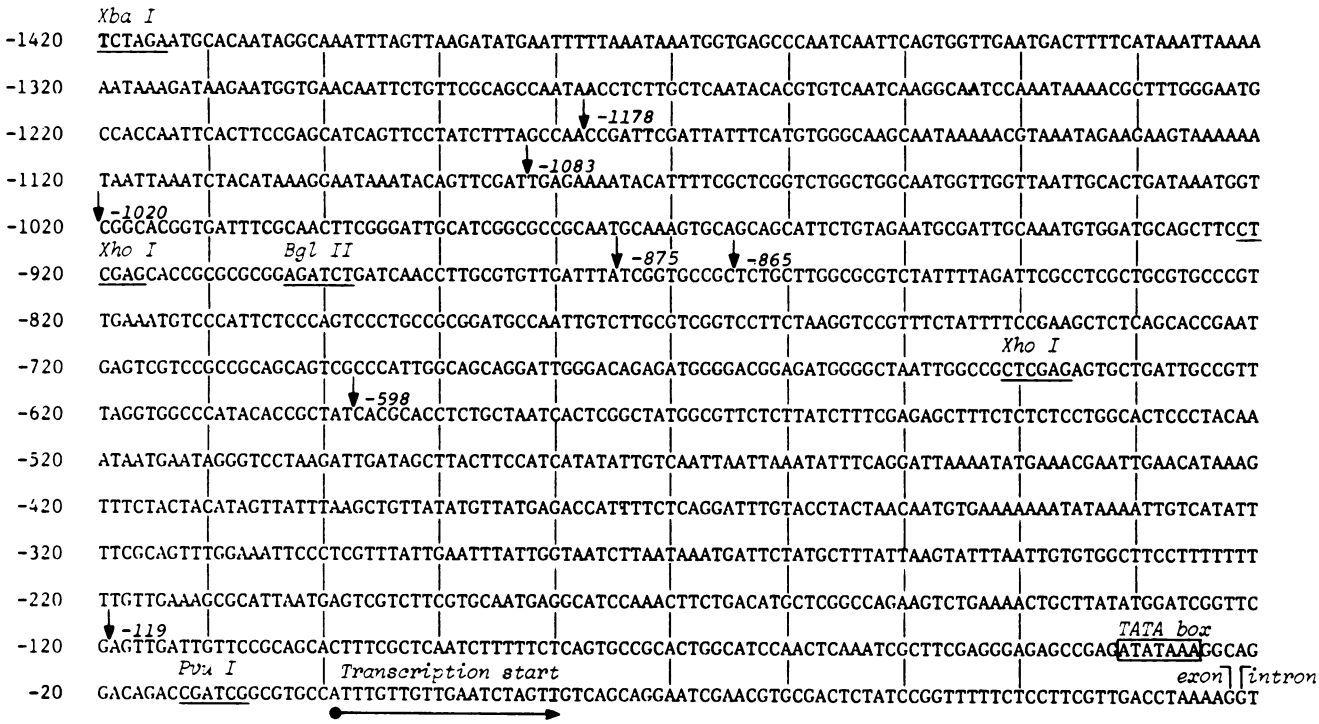

FIG. 8. Sequence of the 5'-flanking region of *act88F*. The illustrated sequence includes 1,420 nucleotides of 5'-flanking sequence, as well as the first exon. Arrows denote borders of deletions described in the text. Also shown are positions of *Xba*I, *Xho*I, *Bgl*II, and *Pvu*I restriction sites.

those having 598 nucleotides had no detectable function. Clearly, the 5' border of an essential element lies near or between these breakpoints. Accordingly, XhoDel, which lacked all sequence between −919 and −640, accumulated *act88F* mRNA at reduced levels, demonstrating that essential sequences lie within this region. However, XhoDel transcripts were both relatively abundant (compared with those of an "average" *Drosophila* gene) and tissue specific, demonstrating that one or more regulatory elements remain intact. Therefore, despite the fact that the developmental program of the *act88F* gene is comparatively simple, its transcription is probably governed by more than one regulatory element.

None of our deleted or reconstructed alleles affected the temporospatial pattern of *act88F* mRNA accumulation. The significance of that observation is difficult to interpret without further experimentation, although several salient points can be cited. Our results could be most simply interpreted to mean that XhoDel lacked a constitutively acting upstream enhancer of transcription, while leaving other tissue-specific regulatory elements unaffected. Such tissue-specific elements could in theory reside near where transcription is initiated, within the transcribed region, or within 3'-flanking sequences. Recent results (9; our laboratory) have delimited the region in which the tissue-specific regulatory element(s) resides. Hiromi et al. constructed a fusion gene in which the 5'-flanking (to −902) and untranslated region, first intron, and codons 1 through 85 are joined to the β-galactosidase gene of *Escherichia coli*. This construct drives the accumulation of enzyme only in flight muscles. Our laboratory has constructed and tested a more extreme derivative in which only the 5'-flanking region and first exon (truncated after nucleotide +32) have been joined to the structural gene for *Drosophila* alcohol dehydrogenase (J. Biggs and E. Fyrberg, unpublished results). Like Hiromi et al., we observed that enzyme accumulated only within flight muscles. These results, together with data presented in this manuscript, suggest that the tissue-specific regulatory region resides between −600 and the transcription initiation point. However, this possibility must be rigorously tested by inserting portions of this sequence into a heterologous reporter gene and subsequently determining whether transcripts accumulate only within flight muscles.

A final topic warranting discussion is the use of *act88F* gene fusions. As we have demonstrated, the 5'-flanking region of *act88F* (Fig. 8) is compact, occupying only 1,000 nucleotides. It should be possible to use this sequence to drive the accumulation of a variety of gene products within a tissue which is not essential for viability, the indirect flight muscles. One could, for example, fuse this regulatory region to the protein-coding region of an easily recognizable and selectable catalytic function, for example, *Drosophila* alcohol dehydrogenase, and introduce the fusion gene into an *Adh*-null strain by p-element-mediated transfer. Such a tester strain might facilitate the isolation of mutant alleles of gene encoding *trans*-acting effectors of *act88F* gene activity and thus facilitate further investigations of the genetic circuitry which regulates flight muscle development.

ACKNOWLEDGMENTS

We thank Barry Yedvobnick and Spyros Artavanis-Tsakonas for making available an adult *Drosophila* cDNA library and Christine Karlik for isolating and sequencing the full-length *act88F* cDNA.

This work was supported by grants from the National Institutes of Health and Muscular Dystrophy Association to E.A.F. and by an American Cancer Society Postdoctoral Fellowship to P.K.G.

LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic

acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
2. **Banerji, J., L. Olson, and W. Schaffner.** 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell **33**:729–740.
3. **Banerji, J., S. Rusconi, and W. Schaffner.** 1981. Expression of a β-globin gene is enhanced by remote SV40 DNA sequencs. Cell **27**:299–308.
4. **Bourouis, M., and G. Richards.** 1985. Remote regulatory sequences of the *Drosophila* glue gene *sgs 3* as revealed by P-element transformation. Cell **40**:349–357.
5. **Ephrussi, B., and G. W. Beadle.** 1936. A technique of transplantation for *Drosophila*. Am. Nat. **70**:218–225.
6. **Fyrberg, E. A., J. W. Mahaffey, B. J. Bond, and N. Davidson.** 1983. Transcripts of six *Drosophila* actin genes accumulate in a stage and tissue specific manner. Cell **33**:115–123.
7. **Heberlein, U., B. England, and R. Tjian.** 1985. Characterization of *Drosophila* transcription factors that activate the tandem promoters of the alcohol dehydrogenase gene. Cell **41**:965–977.
8. **Hiromi, Y., and Y. Hotta.** 1985. Actin gene mutations in *Drosophila*: heat shock activation in the indirect flight muscles. EMBO J. **4**:1681–1687.
9. **Hiromi, Y., H. Okamoto, W. J. Gehring, and Y. Hotta.** 1986. Germ line transformation with Drosophila mutant actin genes induces constitutive expression of heat shock genes. Cell **44**:293–301.
10. **Holmgren, R., V. Corces, R. Morimoto, R. Blackman, and M. Meselson.** 1981. Sequence homologies in the 5′ regions of four Drosophila heat shock genes. Proc. Natl. Acad. Sci. USA **78**:3775–3778.
11. **Karess, R. E., and G. M. Rubin.** 1984. Analysis of P transposable element functions in *Drosophila*. Cell **38**:135–146.
12. **Karlik, C. C., M. D. Coutu, and E. A. Fyrberg.** 1984. A nonsense mutation within the 88F actin gene disrupts myofibril formation in *Drosophila* indirect flight muscle. Cell **38**:711–719.
13. **Levis, R., T. Hazelrigg, and G. M. Rubin.** 1985. Separable cis-acting control elements for expression of the white gene of Drosophila. EMBO J. **4**:3489–3499.
14. **Mahaffey, J. M., M. D. Coutu, E. A. Fyrberg, and W. Inwood.** 1985. The flightless mutant *raised* has two distinct genetic lesions affecting accumulation of myofibrillar proteins in flight muscle. Cell **40**:101–110.
15. **Mason, P., L. M. C. Hall, and J. Gausz.** 1984. The expression of heat shock genes during normal development in *Drosophila melanogaster*. Mol. Gen. Genet. **194**:73–78.
16. **Maxam, A., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.

16a. **Okamoto, H., Y. Hiromi, E. Ishikawa, T. Yamada, K. Isoda, H. Maekawa, and Y. Hotta.** 1986. Molecular characterization of mutant actin genes which induce heat shock proteins in *Drosophila* flight muscles. EMBO J. **5**:589–596.

17. **Picard, D.** 1985. Viral and cellular transcription enhancers. Oxford Surveys Eukaryotic Genes **2**:24–48.
18. **Pirotta, V., H. Steller, and M. P. Bozzetti.** 1985. Multiple upstream regulatory elements control the expression of the *Drosophila white* gene. EMBO J. **4**:3501–3508.
19. **Rubin, G. M., and A. C. Spradling.** 1983. Vectors for P element mediated gene transfer in *Drosophila*. Nucleic Acids Res. **11**:6341–6351.
20. **Sanchèz, F., S. L. Tobin, U. Rdest, E. Zulauf, and B. J. McCarthy.** 1983. Two *Drosophila* actin genes in detail: gene structure and transcription during development. J. Mol. Biol. **163**:533–551.
21. **Snyder, M., M. Hunkapiller, D. Yuen, D. Silvert, J. Fristrom, and N. Davidson.** 1982. Cuticle protein genes of *Drosophila*: structure, organization and evolution of four clustered genes. Cell **29**:1027–1040.
22. **Spradling, A. C., and A. P. Mahowald.** 1979. Identification and genetic localization of mRNAs from ovarian follicle cells of *Drosophila melanogaster*. Cell **16**:589–598.
23. **Spradling, A. C., and G. M. Rubin.** 1982. Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science **218**:341–347.
24. **Spradling, A. C., and G. M. Rubin.** 1983. The effects of chromosomal position on the expression of the *Drosophila* xanthine dehydrogenase gene. Cell **34**:47–57.