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Cloning of a gene localized and expressed at the ecdysteroid regulated puff 74EF in salivary glands of *Drosophila* larvae

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The puffing cycle of salivary gland chromosomes of *Drosophila* larvae, which initiates the developmental path to pupariation, is induced by ecdysteroid hormone. Its action leads to prominent puffs at loci 2B5, 74EF and 75B. Fragments of the 74EF puff of the *D. melanogaster* 3L chromosome were microdissected from salivary gland squashes. *Eco*RI-digested DNA of these fragments was cloned into λ phage. Clones were screened with puff stage-specific cDNA probes. Thirteen out of 650 clones hybridized preferentially with puff stage 4-specific cDNA. The prominent early puffs at 74EF and 75B are most active between puff stage 4 and 6. Therefore, one of the 13 λ phages was chosen for further analysis. It was used to isolate 24 kb of *Drosophila* DNA from genomic libraries. The DNA hybridized *in situ* to locus 74F. The 74F DNA coded for a transcript, which was made in salivary glands, but not in fat body of third instar larvae. It accumulated in K_c cells in response to ecdysteroid treatment. The polyadenylated transcript size was ~2.7 kb as judged by Northern blot analysis. The transcription start site of the 74F gene has been mapped. Sequences upstream of the transcription site contain several sequence elements common to other eucaryotic genes, including potential Z-DNA forming sequences. Also, there is sequence homology to upstream sequences, which have been involved in the regulation of transcription of the salivary gland glue protein 4 gene.

Key words: microdissection/poly(A)$^+$ RNA/*in situ* hybridization/DNA sequencing

Introduction

Developmental programs are based upon temporal and sequential gene activities. The ecdysteroid-induced pupariation of 3rd instar *Drosophila* larvae is well suited for studying the molecular basis of the sequential and transient activity of genes as development proceeds. Several larval tissues have polytenized interphase chromosomes (Beermann, 1962), which are readily visualized under the light microscope. Specific, local decondensations in the structure of these interphase chromosomes (puffs) are the visible manifestations of active genes (Beermann, 1972). Changes in puffing activity of polytene chromosomes of salivary glands of third instar *Drosophila* larvae are initiated as a consequence of an ecdysteroid stimulus (Clever, 1961; Berendes, 1967; Ashburner, 1972). The relative timing and nature of the response of puffs to ecdysteroid hormone has been extensively studied (Ashburner, 1972, 1973). In general, there is a good correlation between the temporal sequence of puffing activities induced by ecdysteroid hormone and the binding of hormone to these puffs (Dworniczak *et al.*, 1983). The sequence of puffing activities commences at 'early' puffs, which are induced by ecdysteroid within minutes (Ashburner *et al.*, 1974). Prominent 'early' puffs have been observed at loci 2B5, 74EF and 75B (Ashburner, 1972). The temporal and sequential binding of ecdysteroid to chromosomal loci during the larval/prepupal puffing cycle suggested that additional factors besides steroid hormone are necessary for controlling the temporal sequence of gene activations during development from larvae to prepupae (Ashburner *et al.*, 1974; Dworniczak *et al.*, 1982). Genes at the early puffs, at least in part, encode such controlling factors, which are necessary for the larval/prepupal puffing cycle to proceed.

Mutations within locus 2B5 influence the development affecting the processes of hormonal induction (Belyaeva *et al.*, 1981). The complex overlapping complementation map of these mutants indicated a complex informational organisation in this puff. Three genes are apparently in the region 2B3-4–2B7-8, which affect the hormonal induction of the puffing sequence which leads to pupariation (Belyaeva and Zhimulev, 1982). Mutations for the early puffs at 74EF and 75B are not available. However, genotypes aneuploid for the 74EF/75B puffs have been constructed (Walker and Ashburner, 1981). In the duplication genotype the 74EF/75B puffs are active for less time than in the euploids while in the deficient genotype they are active for a longer period. This suggests that 74EF/75B puffs remain active for as long as is necessary to code for the synthesis of a pre-determined amount of their product(s), which is used for the control of puffing later on in the larval/prepupal puffing cycle. Gene(s) in these early puffs apparently comprise regulatory elements for the larval/prepupal puffing cycle and thus for the developmental processes, which take place in salivary glands towards the end of larval life and the beginning of metamorphosis. Therefore, we were interested in isolating the DNA which is contained in early puffs. This should open an avenue for characterizing the genes of early puffs and the molecular basis of their products.

Recently, a microcloning technique has been developed for isolating recombinant clones from microdissected fragments of squash preparations of salivary gland chromosomes (Scalenghe *et al.*, 1981; Pirrotta *et al.*, 1983). In the present work, we have used one clone, which was obtained by microdissection, to isolate a continuous array of 24 kb of genomic DNA, which is part of the 74F locus. It contains a developmentally regulated gene expressed in salivary glands of *Drosophila* larvae. The content of the RNA, which is encoded in this gene, is under hormonal control in *Drosophila* tissue culture cells.

Results

Our strategy for isolating DNA molecules containing ecdysteroid-regulated *D. melanogaster* gene sequences of

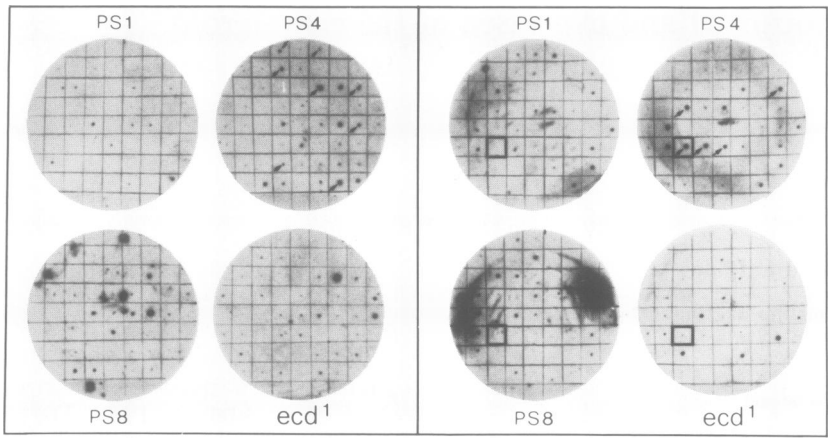

Fig. 1. Recombinant phages were constructed from locus 74EF of polytene chromosomes of *D. melanogaster* salivary glands by a microcloning technique (Scalenghe *et al.*, 1981). Phages of small lysates were dotted onto nitrocellulose filters. Each dot contained 5 x 10^7 phage. After denaturing with alkali, they were neutralized and baked *in vacuo* for 2 h, the filters were then hybridized with ^{32}P-labeled cDNA. cDNA was prepared by reverse transcription of poly(A)$^+$ RNA, isolated from salivary glands of 3rd instar larvae of *D. melanogaster* Oregon R or *ecd*^{1} *Drosophila* mutants (Garen *et al.*, 1977). PS1 and PS8 probes were obtained from poly(A)$^+$ RNA extracted from salivary glands of appropriate puff stages. The PS4 probe was prepared from poly(A)$^+$ RNA obtained from PS1 salivary glands which had been incubated for 2 h in the presence of 5 x 10^{-6} M ecdysterone and 7 x 10^{-4} M cycloheximide. Arrows indicate clones which preferentially hybridized to the PS4 cDNA probe. The boxed clone represents λ HD 513 which was used in subsequent studies of locus 74F.

locus 74EF of the third chromosome was to construct a recombinant DNA library with *D. melanogaster* DNA fragments cut out from salivary gland chromosomes with micromanipulation techniques, as described previously (Scalenghe *et al.*, 1981). Locus 74EF spans ~200 kb of the *D. melanogaster* genome. The puff at 74EF was microdissected from 15 squashed chromosomes briefly fixed in 45% acetic acid. Chromosomal fragments were transferred into a 1 nl droplet suspended over an oil-filled chamber. Chromosomal proteins were digested with 0.05% proteinase K in the presence of SDS followed by phenol extraction. Extracted DNA fragments were then cleaved by *Eco*RI to generate restriction fragments for *in vitro* packaging into λ phage (λ 641) containing a single *Eco*RI site in the 434 immunity region (Murray, 1983). This λ vector can accept inserts from 0 to 10 kb and, therefore, is suited to clone the *Eco*RI-digested chromosomal DNA, which should consist of fragments of an average size of 3 kb (Pirotta *et al.*, 1983). In fact, an analysis of the recombinant phages, which were obtained, showed that most of them contained *Drosophila* DNA fragments smaller than 1 kb (data not shown). Recombinant λ phages were detected, at first, by their plaque morphology. 650 clones were obtained with the starting material of 15 dissected chromosome fragments. The clones were screened with a ^{32}P-labeled cDNA probe prepared from poly(A)$^+$ RNA of salivary glands of 3rd instar larvae of *D. melanogaster*. 80 of the 650 clones contained sequences which were complementary to poly(A)$^+$ RNA of 3rd instar larval salivary glands. These 80 clones were selected for a second screen. Since we were interested in genes of early puffs, the second screen was carried out with cDNA probes, prepared from poly(A)$^+$ RNA of larval salivary glands of different puff stages. The puffing pattern of intermolt glands is described as puff stage 1 (PS1) (Ashburner, 1972). Early puffs are most active around PS4. At PS8, early puffs have regressed and are inactive, now 'late' puffs are active. PS1-, PS4- and PS8/10-specific cDNA probes were used in the second screen for determining which of the 80 clones contained DNA sequences complementary to poly(A)$^+$ RNA enriched in salivary glands of PS4. Details of the preparation of these probes will be described elsewhere (W.Koerwer and O.Pongs, in preparation). The differential screen with puff stage-specific cDNA probes was extended with another cDNA probe, which was prepared from poly(A)$^+$ RNA of salivary glands of *ecd*^{1} mutants (Garen *et al.*, 1977). At non-permissive temperature, early or late puffs are not active in the polytene chromosomes of larval salivary glands of *ecd*^{1} mutants (Dworniczak *et al.*, 1982). Therefore, poly(A)$^+$ RNA in the mutant glands should not be complementary to genes of early puffs.

Lysates of the 80 recombinant λ phages, selected from the first screen, were dotted onto nitrocellulose filters in replica. The dots were hybridized with the PS1, PS4, PS8 or *ecd*^{1} cDNA probe. Thirteen clones were obtained, as indicated by arrows in Figure 1, which preferentially hybridized with the PS4 cDNA probe. They hybridized weakly or beyond the limits of detection with the other cDNA probes. These clones were, therefore, prime candidates for gene(s) active at early puff stages. Three of the 13 clones were hybridized back individually to the original mini-library constructed from the microdissected region. They were represented only once. This roughly indicates a low degree of redundancy of this library. One of the 13 clones (λ HD 513), which is boxed in Figure 1, was selected for further analysis. It contained two *Eco*RI *Drosophila* DNA inserts of 0.8 and 2.5 kb (Figure 3A). It was the largest insert present in the 13 recombinant λ phages. The chromosomal location of λ HD 513 DNA was verified by *in situ* hybridization with ^{3}H-labeled, nick-translated DNA probes. Figure 2 shows that the 0.8-kb fragment of λ HD 513 originated from locus 74F. The 2.5-kb fragment hybridized *in situ* at the same locus. Hybridization of nick-translated, ^{32}P-labeled λ HD 513 DNA to Southern blots of *Eco*RI and *Hind*III digests of *Drosophila* DNA indicated that the DNA was a unique sequence within locus 74F (data not shown). Thus, λ HD 513 DNA originated from a gene located in an early ecdysteroid regulated puff, which codes for poly(A)$^+$ RNA in 3rd instar salivary glands.

λ Clones of a random shear *Drosophila* recombinant DNA

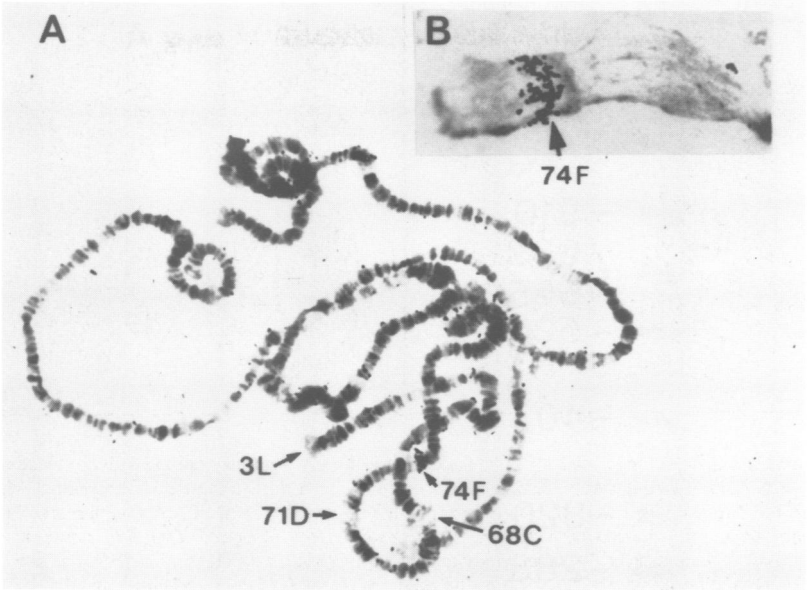

Fig. 2. In situ hybridization (Rigby *et al.*, 1977) of the 0.8-kb ^{3}H-labeled *Eco*RI fragment of λ HD 513 to a squash preparation of polytene chromosome of 3rd instar larval salivary glands of *D. melanogaster* **(A)**. A stretched squash preparation of the 74EF/75B region is shown in the insert **(B)**. Exposure time was 8 days. 68C and 71D indicate landmarks on chromosome 3L.

genomic library (Maniatis *et al.*, 1978) were screened for sequences complementary to λ HD 513. One genomic clone was obtained with a 13-kb *Drosophila* DNA insert (λ MOT-1) encompassing the two *Eco*RI fragments of λ HD 513. A detailed restriction analysis of λ MOT-1 revealed that the λ HD 513 *Eco*RI fragments represented a continuous segment of *Drosophila* genomic DNA (Figure 3A). Another genomic library of *Drosophila* DNA (given to us by H.Jaeckle, Tübingen), which had been prepared by cloning *Sau*3a partial digests of *Drosophila* DNA into λ vector, yielded four additional clones (λ MOT 2, 3, 4, 10 in Figure 3A). These five λ MOT 513 clones encompassed a total of 24 kb of the *Drosophila* genome (Figure 3A). The direction of transcription of the 74F gene, encoded in this stretch of DNA, was determined by the experiment shown in Figure 3B. The two strands of λ MOT-1 were separated by gel electrophoresis. The 5′- to 3′-direction of each strand was determined by hybridizing strand-specific probes to Southern blots of the gel. Then, the direction of transcription was simply determined by identifying the strand of λ MOT-1 DNA which could hybridize with a [^{32}P]cDNA probe. Figure 3B shows that this cDNA probe hybridized to the strand whose 5′ and 3′ direction is from right to left, which must therefore be the direction in which the 74F gene is transcribed. The transcription start site was mapped by S1 nuclease protection experiments (Berk and Sharp, 1978). Hybridizations with ^{32}P-labeled cDNA probe to Southern blots of an *Eco*RI digest of λ MOT-1 had indicated that besides the 0.8-kb *Eco*RI fragment of λ HD 513 the 4.5-kb *Eco*RI fragment to the left also contained coding sequences. Since the coding strand as shown in Figure 3B was the 3′ to 5′ strand of λ MOT-1, the transcription start site of the 74F gene had to be within λ HD 513 DNA. Therefore, the 0.8-kb *Eco*RI and the 0.8-kb *Eco*RI-*Kpn*I fragment of λ HD 513 (fragments I and II, respectively, in Figure 3A) were subcloned into plasmid vector pUC8 (Vieira and Messing, 1983). Both fragments were isolated from the subclones and were ^{32}P end-labeled at their leftward 5′ side. They were

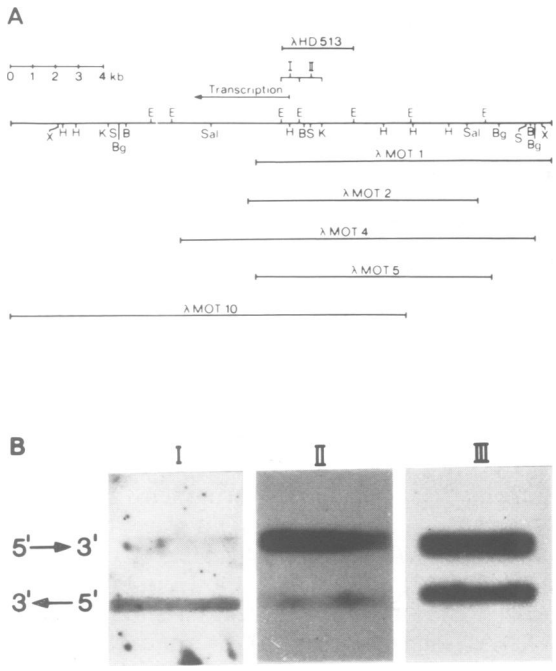

Fig. 3. (A) Restriction map of the cloned 74F region. The distance is measured in kb starting from the left end, which is nearest to the chromocenter (M.Rentrop and O.Pongs, unpublished). *Eco*RI = E, *Bam*HI = B, *Hind*III = H, *Sal*I = Sal, *Kpn*I = K, *Xba* = X, *Bgl*II = Bg, *Sma*I = S. The direction of transcription is indicated by an arrow. I and II designate the 0.8-kb *Eco*RI fragment and the 0.8-kb *Eco*RI-*Kpn*I fragment, which were used in Southern/Northern and S1 mapping experiments (Figures 4–6). **(B)** Strands of λ MOT-1 were separated after alkali treatment by electrophoresis on a neutral 0.5% agarose gel (Maniatis *et al.*, 1982). Gel pieces containing the single-stranded DNAs were blotted on nitrocellulose. The blot was hybridized to [^{32}P]cDNA prepared from poly(A)$^{+}$ RNA of *Drosophila* **(I)**, a 13-kb *Bam*HI-*Eco*RI fragment of the left arm of λ vector charon 4A (Maniatis *et al.*, 1982) which was 5′-labeled at the *Eco*RI side **(II)** or to λ HD MOT-1 **(III)**, which was ^{32}P-labeled by nick-translation. For further details see Materials and methods.

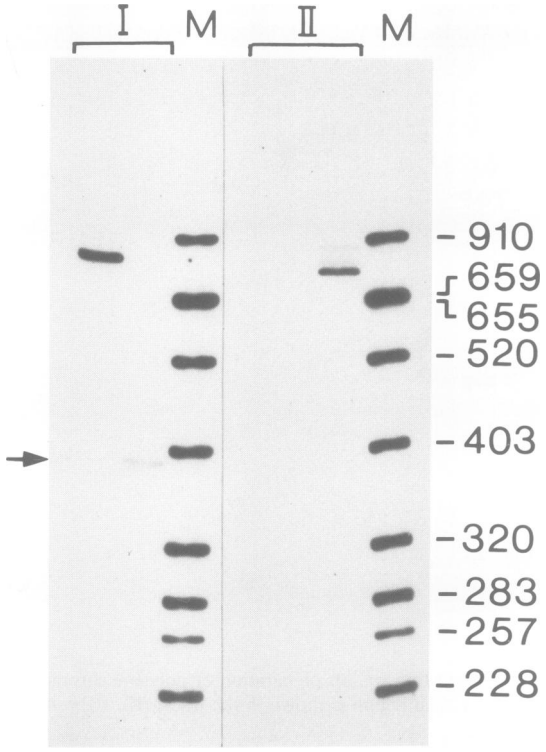

Fig. 4. Determination of the transcription start site of the 74F gene by S1 nuclease protection experiments (Berk and Sharp, 1978). Fragments I and II, indicated in Figure 3A, were ^{32}P-end-labeled on the left 5′ side. They were hybridized to 100 μg of poly(A)$^+$ RNA of *D. melanogaster*. After digestion, S1 nuclease-protected DNA was separated on a denaturing 4% acrylamide gel containing 8 M urea (Maxam and Gilbert, 1977). **Lanes I** show the 0.8-kb *Eco*RI fragment of λ HD 513 on the left before, and on the right after, S1 nuclease digestion. The arrow indicates the S1 nuclease-protected 400 bp long fragment. **Lanes II** show the 0.8-kb *Eco*RI-*Kpn*I-fragment of λ HD 513 on the left after, and on the right before, S1 nuclease digestion. Nucleotide lengths of marker DNA (M) are indicated on the right.

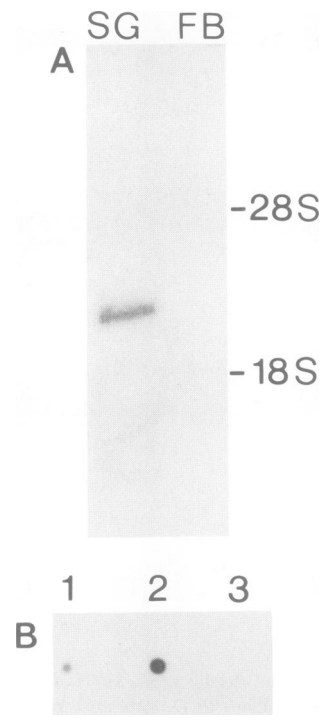

Fig. 5. Analysis of salivary gland and of fat body RNA for 74F gene sequence. PS2−4 *D. melanogaster* larvae were dissected. Both, salivary glands and fat body were collected for RNA extraction. **(A)** 2 μg of salivary gland RNA (SG) and of fat body RNA (FB) were electrophoresed on an agarose gel containing 2.2 M formaldehyde. A Northern blot of the gel was hybridized to the ^{32}P-labeled, nick-translated 0.8 kb *Eco*RI-fragment of λ HD 513. Marker RNA was HeLa rRNA. Exposure time for autoradiography was 8 days. **(B)** 2 μg each of total salivary gland RNA **(1)** poly(A)$^+$ RNA **(2)** or poly(A)$^-$ RNA **(3)** were dotted on nitrocellulose and were hybridized as above with the ^{32}P-labeled, nick-translated 0.8-kb *Eco*RI fragment of λ HD 513.

hybridized with total *Drosophila* poly(A)$^+$ RNA. Single-stranded regions were removed from the hybridization products by S1 nuclease digestion. The S1 nuclease-resistant hybrids were denatured and electrophoresed in a sequencing gel (Figure 4). The end-labeled *Eco*RI-*Kpn*I fragment II was completely digested, whereas 400 ± 5 nucleotides of the end-labeled *Eco*RI fragment I were protected from S1 nuclease digestion by RNA hybridization. This places the mRNA start point within the 0.8-kb *Eco*RI fragment, very near to the left of the *Hind*III restriction site as shown in Figure 3A.

We determined the size of the mRNA, transcribed at 74F. Total RNA isolated from PS2−4 salivary glands of *D. melanogaster* larvae, was separated by electrophoresis in agarose gels containing formaldehyde, and thereafter blotted to nitrocellulose (Thomas, 1980). The Northern blots were hybridized with ^{32}P-labeled nick-translated 0.8-kb *Eco*RI fragment (I). The probe hybridized to a ~2.7 kb long polyadenylated RNA (Figure 5A, B). S1 nuclease protection experiments with the *Eco*RI/*Sal*I fragment of λ MOT 10 (Figure 3A) and the *Sal*I/*Eco*RI fragment of λ MOT 4 (Figure 3A) showed that the coding sequence is uninterrupted (data not shown). This places the 3′ boundary of the RNA-coding region near the *Sal*I site in λ MOT 4 (Figure 3A). RNA, which was isolated from larval fat body, the predominant tissue of early 3rd instar larvae, did not contain a species, which was complementary to our 74F DNA probe (Figure 5A). These data indicate that in *Drosophila* larvae of early 3rd instar, the cloned 74F gene is not expressed in fat body cells, where ecdysteroid also induces a puff at locus 74EF (Richards, 1982).

Early puffs are induced by ecdysteroid hormone (Ashburner, 1972). Since we had cloned a gene located in the early puff at 74F, it was important to know how transcription of this gene responded to hormonal stimulus. The low abundance of the 74F gene transcript made it very difficult to measure RNA synthesis in salivary glands of *D. melanogaster* larvae. Therefore, we turned to *Drosophila* tissue culture cells, which are ecdysterone sensitive (Maroy *et al.*, 1978). Tissue culture cells were treated with 5 x 10^{-6} M 20-hydroxyecdysone. RNA was extracted from the tissue culture cells. Hybridization of R dots of poly(A)$^+$ RNA with ^{32}P-labeled nick-translated 0.8-kb *Eco*RI fragment was positive. This suggested that K_c cells produced 74F mRNA. The transcript had the same size as that in salivary glands within the limits of our Northern blot analysis (Figure 6). *Drosophila* tissue culture cells were treated from 0 to 8 h with 5 x 10^{-6} M 20-hydroxyecdysone. At hourly intervals total RNA was extracted for Northern blot analysis (Figure 6). The result shows that the content of 74F mRNA in *Drosophila* tissue culture cells steadily increased to reach a plateau at ~6 h in response to ecdysteroid. Tissue culture cells which were not incubated with ecdysterone did not contain 74F mRNA. As shown in Figure 6 lane 4C, incubation of tissue culture cells with

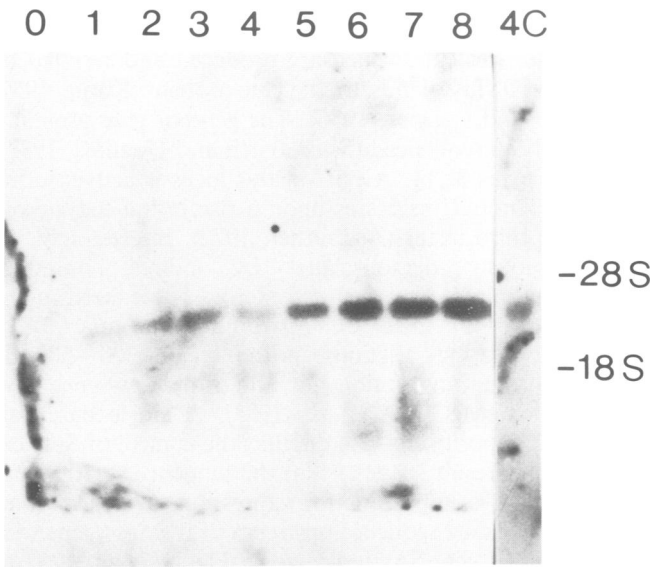

Fig. 6. Content of 74F mRNA in *Drosophila* tissue culture cells in response to ecdysteroid. K_c cells were incubated with 5 x 10^{-6} M 20-hydroxyecdysone. At hourly intervals, aliquots were removed for RNA extraction. In parallel, cells were treated with 5 x 10^{-6} M 20-hydroxyecdysone and 7 x 10^{-4} M cycloheximide for 4 h (**lane 4C**). 10 μg of each RNA preparation was electrophoresed on a formaldehyde-containing gel, blotted and hybridized to ^{32}P-labeled, nick-translated 0.8-kb *Eco*RI fragment of λ HD 513. **Lanes 0–8** are numbered according to the hours of ecdysterone treatment. Marker RNA was HeLa rRNA. Exposure time for autoradiography was 8 days.

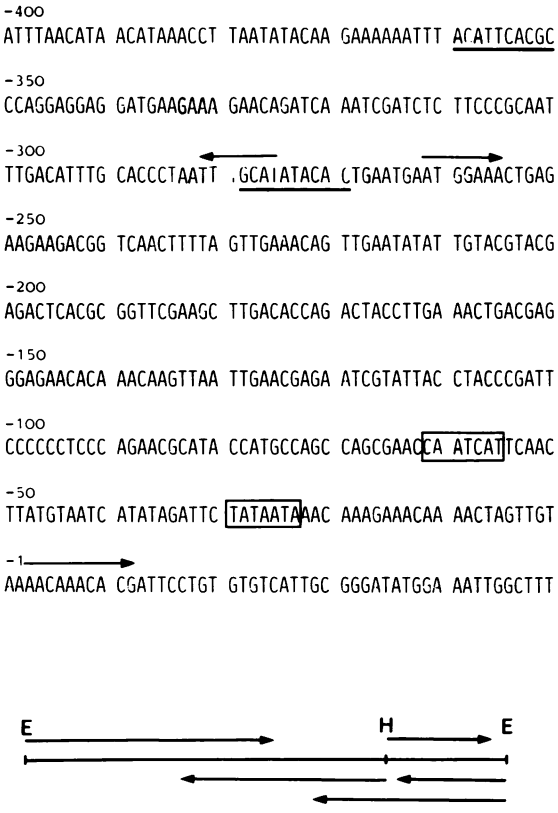

Fig. 7. DNA sequence upstream of the 74F gene. The −1 position was assigned to the first base upstream from transcription initiation. The arrow indicates the direction of transcription. 'TATA' and 'CAAT' box are framed. Potential Z-DNA forming sequences are underlined by black bars. The broken arrows point out an inverse repeat. An isomer of this repeat structure is found in the sequence upstream of the *Sgs4* gene (McGinnis *et al.*, 1983). The lower part indicates the restriction fragments of λ HD 513, which were used for sequencing according to Maxam and Gilbert (1977). *Eco*RI = E, *Hind*III = H.

20-hydroxyecdysone in the presence of cycloheximide did not inhibit the hormonal induction of 74F mRNA. This suggests that the ecdysterone stimulus is probably a primary effect on the synthesis of 74F mRNA, although our results cannot exclude alternative interpretations. In any case, these results agree well with the observation in Figure 1, that λ HD 513 complementary RNA is enriched in PS4 salivary glands. This indicates that ecdysteroid induction of an early puff at 74F results in an increased production of λ HD 513-74F RNA.

400 nucleotides upstream of the TATA box at the 5′ end of the 74F gene have been sequenced (Figure 7). The sequence has several features which are known for many eucaryotic genes. The boxed sequence at −57 to −63 is similar to the 'CAAT' box, noticed at this approximate position upstream from the mRNA start point of other eucaryotic genes (Breathnach and Chambon, 1981). The underlined sequences at −270 and −352 are homologous to potential Z-DNA-forming sequences in enhancer elements (Nordheim and Rich, 1983), which are found as pairs with nucleotide sequences between them varying in length from ~50 to 80 bp. The arrows in Figure 7 point out an inverse repeat, which has an interesting relationship to a regulatory element of the *Sgs4* gene at locus 3C11 (McGinnis *et al.*, 1983), as is discussed below.

Discussion

The puff at 74EF is a typical ecdysteroid puff in the sense that its activity is sharply increased immediately after the contact of salivary glands of 3rd instar larvae with ecdysteroid hormone (Ashburner *et al.*, 1974). However, the large size of this puff makes it difficult to decide how many bands it originates from. The whole region spans at least 10 bands according to the chromosome map of Bridges (1935). Ecdysteroid hormone was bonded to this puff upon photoactivation (Dworniczak *et al.*, 1983). In fact, the indirect immunofluorescence micrographs of this puff show two regions (one distal, the other proximal) of intense fluorescence, suggesting that more than one hormone-binding domain is situated in the puff at 74EF (Dworniczak *et al.*, 1982). Contrary to the 2B5-6 puff of *D. melanogaster* (Zhimulev *et al.*, 1981), the 74EF puff has not been studied by the method of saturation with visible or lethal mutations. Using translocations between chromosome three and the Y chromosome, Walker and Ashburner (1981) have constructed aneuploid genotypes that were either duplicate or deficient for the 74EF and 75B early puffs. The observed puffing activities in the aneuploids suggested that the activities of 74EF and 75B are self-regulated, i.e., controlled by the concentration of their own gene products. The cloning of the 74F gene enables us now to identify the nature of these gene products and to study their function at the molecular level. The λ HD 513 clone described in this paper hybridized to the proximal end of the 74EF puff. As this puff is large, it is, of course, not certain that the 24 kb of genomic DNA which has been characterized so far comprises all the information of the 74EF puff which materializes upon ecdysteroid hormone activation. We have found in our mini-library, which was constructed after microdissection, 13 λ phages hybridizing preferentially with PS4-specfic cDNA

probes (Figure 1). If we take into account the low redundancy in this library, more ecdysteroid-inducible transcripts of the 74EF puff will probably be detected. On the other hand, back hybridization of the genomic DNA of the 13 λ phages to *Drosophila* chromosomes has indicated that the transcripts originated from different loci spanning the 72D to 75 region of the third chromosome (T.Möritz and O.Pongs, unpublished data). This result is probably due to imprecise microdissection of the 74EF region. Two of the 13 transcripts originated from 74F.

The PS4-specific transcript, which was complementary to λ HD 513, was characterized by S1 protection experiments (Figure 3) and by Northern blot analysis (Figure 4). The transcription start site was found in the middle of the 0.8-kb *Eco*RI fragment of λ HD 513 and of λ MOT-1. The transcript is polyadenylated and according to the Northern blots has a size of ~2.7 kb. The very long exposure times necessary to obtain positive hybridization signals with nick-translated probes (the 0.8-kb *Eco*RI or 3.5-kb *Sal-Eco*RI fragment of λ MOT-1) suggest that the transcript is very rare. Probes for *Sgs4* mRNA or histone mRNA gave similar signals in 1/20 of the exposure time (data not shown). Ecdysteroid treatment of K_c cells induced an accumulation of the 74F transcript (Figure 6). As the transcript is located in an ecdysteroid-activated early puff, this suggests that the synthesis of the transcript is ecdysteroid regulated, although we cannot be absolutely sure in the absence of kinetic measurements. The puff 74EF is transiently active in the salivary glands. Whether similar transient activity holds for tissue culture cells is doubtful. Further extensive studies are needed to measure synthesis, accumulation and decay of the 74F transcript. At present, we do not have suitable methods to carry out these experiments on single salivary glands of defined puffed stage.

Much is known about ecdysteroid-induced puffing in salivary glands of third instar *Drosophila* larvae due to the establishment of an *in vitro* culture system (Ashburner, 1972). This is difficult to apply to other *Drosophila* tissues which also have polytene chromosomes. Recently, the ecdysteroid-regulated puffing of larval fat body chromosomes has been investigated (Richards, 1982). Among the many notable similarities between puffing patterns of salivary gland and of fat body chromosomes, it is of interest in this context that the early 74EF puff is also observed with fat body chromosomes. Dose response of this puff to ecdysteroid are very similar in both larval tissues (Richards, 1982). Therefore, our Northern blot result (Figure 5) showing that the 74F transcript was made in salivary glands, but not in fat body chromosomes of third instar larvae, was quite unexpected. It indicates that the decondensation of chromatin to form a puff at the same locus does not necessarily result in the transcription of the same gene. It supports the earlier notion that a puff encompasses enough DNA for it to contain genes, which are not functionally related and which are expressed in a tissue-specific manner (Ashburner, 1977). Alternatively, the 74EF puffs in salivary gland and in fat body, although similar in their location, may form from adjacent bands (Zhimulev *et al.*, 1981).

Though a wealth of cytological data has been accumulated over recent years on the phenomenon of ecdysteroid-induced puffing (for reference see Ashburner, 1972; Dworniczak *et al.*, 1982), not much is known about its molecular basis. This paper describes in detail for the first time some properties of a gene located at a prominent early puff site. Thus, we cannot so far compare sequences of the 74F gene with other ecdysteroid-regulated genes of early puffs.

The major proteins, which are produced and secreted by third instar salivary glands are the glue proteins (Korge, 1977; Beckendorf and Kafatos, 1976). The gene of glue protein 4 *(Sgs4)* has been sequenced (Muskavitch and Hogness, 1982). It maps at locus 3C11. A puff at this locus is active during intermolt. The puff regresses upon a rise in the ecdysteroid titer late in third instar (Ashburner, 1972). Interestingly, we have previously demonstrated by indirect immunofluorescence microscopy that ecdysteroid is localized at the intermolt puff of locus 3C (Dworniczak *et al.*, 1983). Naturally occurring deletions in the region 300–500 bp upstream of the transcription start site of the *Sgs4* gene have been mapped (McGinnis *et al.*, 1983). A single base pair substitution at position −344 disrupts the control of *Sgs4* expression. This result suggests that the sequence surrounding position −344 is important for expression. We have compared the published sequence upstream of the *Sgs4* gene with that of the 74F gene. As indicated in Figure 7, at −257 to −284 upstream of the initiation of transcription there is an inverse 7-bp repeat: TTTGCAT − 13 − ATGGAAA. An isomer of this repeat: ATGGAAA − 3 − TACCTTT frames the point mutation at −344 upstream of the *Sgs4* gene (McGinnis *et al.*, 1983). Whether this is coincidental or whether it has some meaning for the different regulation of these genes in 3rd instar larvae is presently not clear.

Materials and methods

Microdissection and microcloning

The details of the procedure are as described by Scalenghe *et al.* (1981). Salivary glands were briefly fixed in 45% acetic acid and squashed. The siliconised coverslip was flicked off after freezing the squash preparation in liquid nitrogen. The squash was washed in absolute ethanol, air dried and mounted in the oil chamber for dissection. Chromosome fragments were cut out with a glass needle using a de Fonbrune micromanipulator. Fragments of 15 chromosomes were collected in a 1 nl aqueous droplet containing 0.5 mg/ml proteinase K and 0.1% SDS. The droplet was extracted with aqueous phenol followed by $CHCl_3$. *Eco*RI (0.2 nl at 120 units/μl) containing 3 x (50 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 mM Tris pH 7.5) buffer was added. After 2 h at 37°C the enzyme was inactivated by placing the oil chamber in a moist Petri dish at 70°C for 20 min. Then, 0.5 nl of *Eco*RI-cut λ 641 DNA (100 μg/ml) in 1 mM ATP containing *Eco*RI buffer was added at 4°C and 0.5 nl of T4 ligase (1–4 units/μl). Ligation was carried out overnight. The ligation products were packed *in vitro* according to Scherer *et al.* (1981).

Genomic libraries

λ MOT clones were isolated from two genomic *Drosophila* DNA libraries. One was prepared and kindly provided by Maniatis *et al.* (1978). This library was constructed from sheared embryonic Canton-S DNA inserted into λ Charon 4 via *Eco*RI linkers. The other library was constructed from partial *Sau*3a digests of embryonic Oregon-R DNA inserted into the λ EMBL4 vector (Murray, 1983). This library was kindly provided by H.Jaeckle (Tübingen, FRG). Restriction fragments of λ MOT phages were subcloned into pUC8 plasmid (Vieira and Messing, 1983). This plasmid has a polylinker region suitable for DNA sequencing according to the Maxam-Gilbert technique (Maxam and Gilbert, 1977). Recombinant DNA was propagated in ER1 host-vector system under L2/B1 containment conditions, as defined in the guidelines of the Federal German Government for recombinant DNA research.

Isolation and labeling of DNA

λ Phage DNA and plasmid DNA were isolated according to Maniatis *et al.* (1982). *In situ* hybridization was carried out with ^{3}H-labeled, nick-translated probes according to Rigby *et al.* (1977). Phage libraries were screened by the method of Benton and Davies (1977). Bacterial colonies were screened according to Grunstein *et al.* (1975). DNA was radioactively labeled by nick translation with [α-^{32}P]ATP (600 Ci/mmol).

Isolation of RNA

RNA of salivary glands and fat body of third instar larvae as well as of K_c

cells was isolated by the CsCl-isothiocyanate method (Gilsin *et al.*, 1974). RNA was stored frozen in 1 mM EDTA, 0.05% SDS, 10 mM Tris (pH 7.4) buffer before further use. Puff stage-specific poly(A)$^+$ RNA, prepared from hand dissected salivary glands of defined puff stages, was a generous gift of W.Koerwer (Bochum, FRG). rRNA of HeLa cells was given to us by B.J.Benecke (Bochum, FRG). ^{32}P-labeled cDNA was prepared by oligo(dT)$_{10-12}$-primed reverse transcription of poly(A)$^+$ RNA (Maniatis *et al.*, 1982). It had a specific activity of 5 x 10^8 c.p.m./μg RNA.

Restriction maps, λ strand separation and sequencing

Restriction maps were derived by a combination of complete, double and partial digests. Partial digests were carried out after end-labelling with polynucleotide kinase followed by gel electrophoresis of the resulting fragments (Maniatis *et al.*, 1982). For nick-translation or for subcloning, fragments were purified from a low melting agarose gel by the method of Tautz and Renz (1983). λ MOT1 strands were separated on a neutral 0.5% agarose gel. The desired bands were cut out and the individual λ strands were transferred to nitrocellulose before hybridization with strand-specific probes. *Drosophila* DNA subcloned into pUC8 was cut with a suitable restriction enzyme, the ends were labeled by filling in with reverse transcriptase followed by a second restriction enzyme cut. The end-labeled restriction fragments were separated on an agarose gel, the desired band was cut out, purified and sequenced as described by Maxam and Gilbert (1977).

Southern and Northern hybridisation

Southern blots were made from agarose gels according to Southern (1975). After baking at 80°C *in vacuo*, nitrocellulose filters were pre-hybridized in 4 x SET, 5 x Denhardt's solution (Denhardt, 1966) at 65°C and then hybridized with labeled probes under the same conditions. Filters were washed four times in 0.1% SDS, 1 x SET at 65°C before autoradiograpy.

For Northern blots, 10 μg RNA were precipitated with ethanol. The pellet was taken up in buffer containing 50% formamide and 2.2 M formaldehyde. RNA was denatured by incubation for 10 min at 50°C. RNA was run on horizontal 1% agarose gels in 20 mM morpholinopropane sulfonic acid, 5 mM Na acetate, 2.2 M formaldehyde. After electrophoresis, the gel was blotted on nitrocellulose filters without prior soaking in 20 x SSC. RNA blots were pre-hybridised for 24 h at 42°C in 50% formamide, 5 x SCC, 0.1% SDS, 1 x Denhardt's solution, 20 mM Na phosphate (pH 7.0), 250 μg/ml denatured salmon sperm DNA, 250 μg/ml yeast tRNA (Thomas, 1980). Blots were hybridised with labeled probes under the same conditions for 36 h at 42°C. Blots were washed four times in 2 x SSC for 5 min at room temperature and three times in 0.1 x SSC, 0.1% SDS for 20 min at 50°C before autoradiography.

S1 protection experiments

^{32}P-End-labeled DNA restriction fragments were co-precipitated with 100 μg of *Drosophila* poly(A)$^+$ and 100 μg yeast tRNA. The pellet was resuspended in 30 μl hybridization mix (400 mM NaCl, 80% formamide, 1 mM EDTA, 40 mM piperazine-1,4-diethane sulfonic acid). The nucleic acids were denatured by incubation at 85°C for 15 min. The hybridization solutions were transferred to 52°C and were kept at this temperature for 3 – 5 h. Following incubation, the tubes were placed on ice and 300 μl of ice-cold S1 digestion buffer [30 mM Na acetate (pH 4.6), 150 mM NaCl, 1 mM Zn acetate, 21 U S1 nuclease] was added, and digestion allowed to proceed for 30 min at 37°C. The reaction was stopped by adding 50 μl of 4 M NH_4 acetate, 100 mM EDTA followed by ethanol precipitation. Supernatants were discarded, the pellets were washed in 75% ethanol, and then dried. The samples were dissolved in 2 μl sequencing gel-loading buffer [80% formamide, 45 mM Tris-borate (pH 8.3), 1 mM EDTA, 0.1% Xylene cyanol, 0.1% bromphenol blue], heated to 90°C for 1 min, chilled, and loaded on sequencing gels to analyze the lengths of S1-protected DNA fragments.

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