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A member of the steroid hormone receptor gene family is expressed in the 20-OH-ecdysone inducible puff 75B in *Drosophila melanogaster*

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ABSTRACT

Drosophila melanogaster DNA has been cloned which encompasses a major part of the 20-OH-ecdysone inducible puff 75B. One 20-OH-ecdysone responsive transcription unit was detected which is expressed into two transcripts which accumulate upon the incubation of salivary glands of 3rd instar larvae with 20-OH-ecdysone. This accumulation is correlated with the 20-OH-ecdysone induced activity of puff 75B. *75B* cDNA analysis indicates that the activity of puff 75B leads to the synthesis of a protein which belongs to the steroid and thyroid hormone receptor superfamily. The highest similarity of the derived *75B* protein sequence was found to the DNA and ligand binding domains of human retinoic acid receptor. A study of the tissue distribution in larvae revealed that *75B* mRNA is present in most, if not all 20-OH-ecdysone target tissues. It is proposed that *75B* protein is a DNA-binding protein playing a key role in mediating the regulation of the larval molt by 20-OH-ecdysone.

INTRODUCTION

The steroid hormone 20-OH-ecdysone plays a key role in the development of *Drosophila melanogaster* (and of insects, in general) [1]. A high 20-OH-ecdysone titre at the onset of pupariation is correlated with temporal and sequential gene activities visible on the polytene chromosomes in salivary glands of 3rd instar *Drosophila* larvae as the induction of a puffing cycle [2]. This puffing cycle has been reproduced *in vitro* by culturing puff stage 1 (PS1) salivary glands in the presence of 20-OH-ecdysone [3]. Prominent features of the puffing cycle are (i) regression of intermolt puffs (e.g. at 68C) (ii) rapid induction of early puffs (e.g. at 74EF and at 75B) and (iii) delayed induction of late puffs (e.g. at 78D) [4]. The gene products of 20-OH-ecdysone induced early puffs are essential for induction of late and for regression of early puffs [5,6]. The sequential and temporal puffing activity seems to involve the presence of 20-OH-ecdysone or hormone receptor complex in active puffs [7].

It is a generally accepted paradigm that the binding of hormone receptor complex to chromosomal sites of early puffs induces their activity and the expression of their respective genes. After the gene products of early puffs have reached a critical concentration, the activity of early puffs ceases and, concomitantly, late puffs become active [5]. Models have been put forward [6,8] which ascribe to the gene products of the early, 20-OH-ecdysone inducible puffs at 74EF and at 75B a central role in the regulation of the puffing cycle. A recent model even proposed that these gene products resemble transcription factors or DNA-binding proteins, respectively [8].

In a previous report, we have described the characterization of a putative transcription factor gene in puff 74EF, the expression of which is regulated by 20-OH-ecdysone [9].

This report describes the characterization of a cDNA corresponding to a 20-OH-ecdysone regulated transcription unit in puff 75B. The deduced protein sequence contains two domains with striking similarities to the DNA and ligand binding domains of members of the steroid and thyroid hormone receptor superfamily. In support of our previous model, we propose that the induction of the early puff at 75B by 20-OH-ecdysone leads to the synthesis of a DNA-binding protein which plays in larval salivary glands a central role for the regulation of the temporal and sequential puffing cycle underlying the onset of metamorphosis.

MATERIAL AND METHODS

Libraries

The method for microdissection and microcloning of 75B DNA has been described [10]. A *Drosophila melanogaster* genomic DNA library was used for isolating TOM phages (OregonR DNA partially digested with Sau3AI and inserted into the BamHI site of EMBL4 [11]). The library was made available to us by H. Jaeckle (München, FRG). cDNA was isolated from two *Drosophila melanogaster* cDNA libraries. Both consisted of oligo(dT) primed cDNA prepared from adult head mRNA of Berlin or of CantonS flies, respectively. cDNA libraries were a gift of E. Buchner (Würzburg, FRG) and P. Salvaterra (City of Hope, USA). Libraries were screened according to Benton and Davis [12].

Cloning of cDNA

Recombinant DNA was manipulated according to Maniatis *et al.* [13] using ERI host vector systems under L1 containment conditions, as defined in the guidelines of the Federal German Government for recombinant DNA research. pBluescript KS^+ or SK^+ (Stratagene) were used as vectors for subcloning.

Isolation of RNA

Salivary glands were hand dissected from 3rd instar *Drosophila melanogaster* OregonR larvae. Salivary glands corresponding to puff stage 1 were incubated *in vitro* according to Ashburner [3]. RNA was isolated from incubated glands by the guanidinium-isothiocyanate method.

Northern Blots

12μg of each salivary gland RNA sample were electrophoretically separated in 1% agarose gels in 2,2 M formaldehyde, 50 mM 3-[N-morpholino]propanesulfonic acid pH 7.0, 1 mM EDTA [14] and transferred to nylon membrane (Nytran, Schleicher and Schuell; Biodyne B, Pall) by overnight diffusion-blotting with 20×SSC. The RNA was then fixed to the nylon membrane by baking for 2h at 80°C. Blots were prehybridized in 50% formamide, 750 mM NaCl, 150 mM Tris/HCl pH 7.9, 5 mM EDTA, 0.1% SDS, 5×Denhardt's solution and 0.1 mg/ml denatured salmon sperm DNA for 2h at 42°C. Hybridization took place under the same conditions for 24−36h with DNA probes labeled with ^{32}P-dCTP by oligonucleotide primed DNA synthesis [15]. Blots were washed in 1×SSC, 0.1% SDS at room temperature followed by several washes in 0.1×SSC, 0.1% SDS at 65°C before autoradiography.

In situ Hybridization

In situ hybridizations of DNA labeled with ^{3}H-dCTP by nick-translation [16] to polytene chromosomes of *Drosophila melanogaster* were as described previously [17]. *In situ* hybridizations to 10μm sagittal sections of paraffin embedded *Drosophila melanogaster* OregonR 3rd instar larvae were according to the procedure described in Hafen *et al.* [18]. ^{35}S-UTP labeled antisense strand RNA probes were obtained from a T3 promoted *in vitro* transcript of *75B* cDNA cloned into the EcoRI site of pBluescript KS^+.

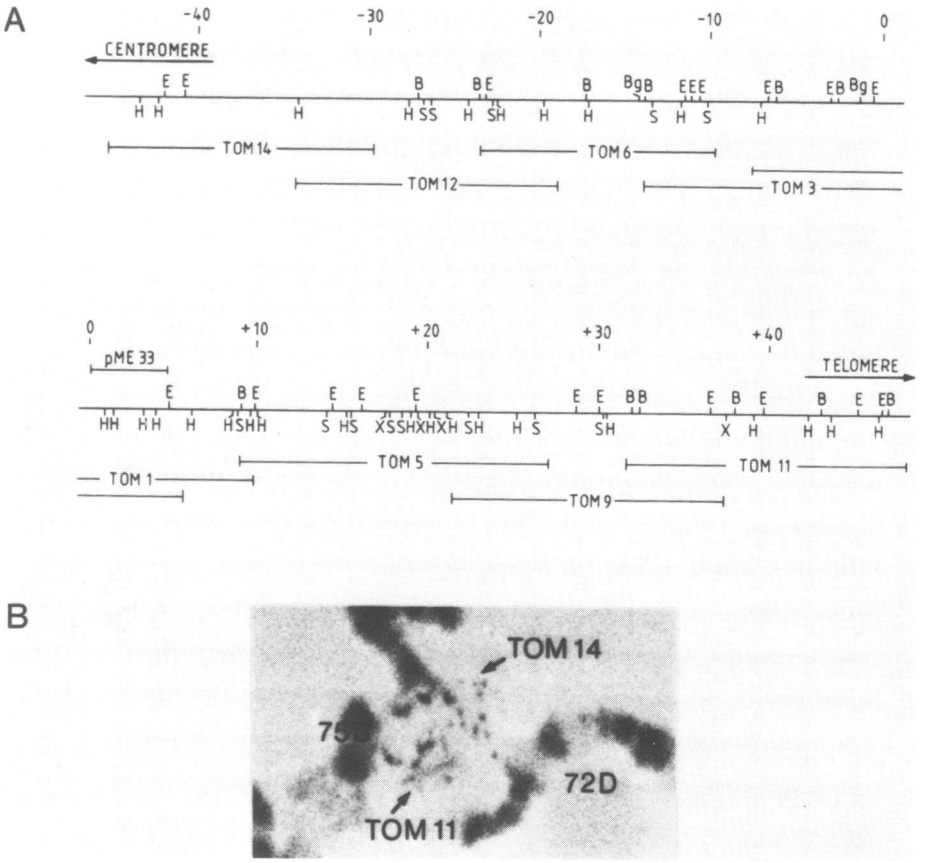


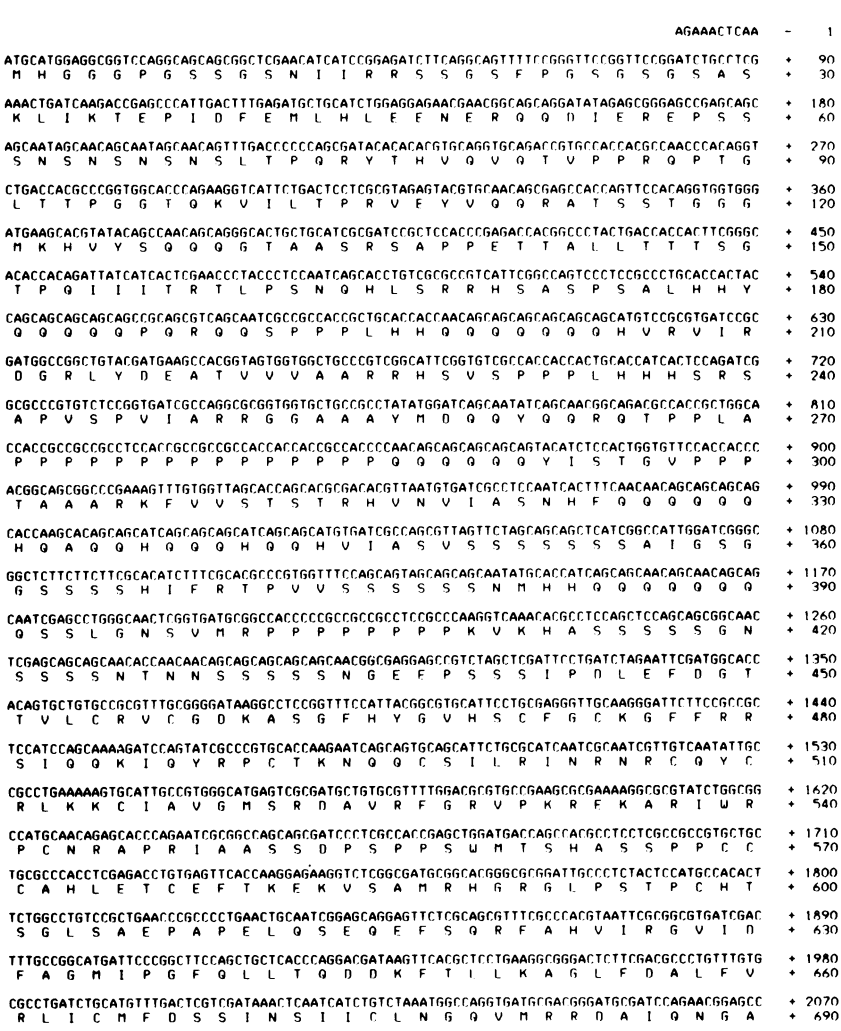
Fig. 1. (A) Restriction map of the isolated 75B genomic DNA. The distance is measured in kb relative to the start site of the chromosomal walk with negative/positive numbers indicating the direction towards the centromere/telomere. Isolated DNA fragments are shown below the restriction map. pME33, the starting clone of the chromosomal walk, was obtained by microcloning 75B DNA. E=EcoRI, B=BamHI, Bg=BglII, H=HindIII, X=XbaI. (B) *In situ* hybridization of ^{3}H-labeled TOM11 and TOM14 DNA to polytene chromosomes. Only the part of third chromosome showing hybridization signals is presented.

DNA sequencing

DNA was sequenced by the dideoxynucleotide chain-termination method [19] employing ^{35}S-dATP and using the Sequenase Kit (United States Biochemicals) or the T7 Sequencing Kit (Pharmacia). Subclones for sequencing were generated either by cloning of restriction fragments or by constructing DNaseI deletion subclones [20].

RESULTS

As previously described for the cloning of DNA in the 20-OH-ecdysone regulated puff at locus 74EF on the 3rd chromosome of *Drosophila melanogaster* [9,10], we have now isolated DNA corresponding to the 20-OH-ecdysone regulated puff at locus 75B by microcloning of microdissected 75B DNA followed by a chromosomal walk. This walk encompassed 100 kb of *Drosophila melanogaster* genomic DNA isolated from an OregonR genomic library as summarized in Fig. 1A. The boundaries as well as the orientation of



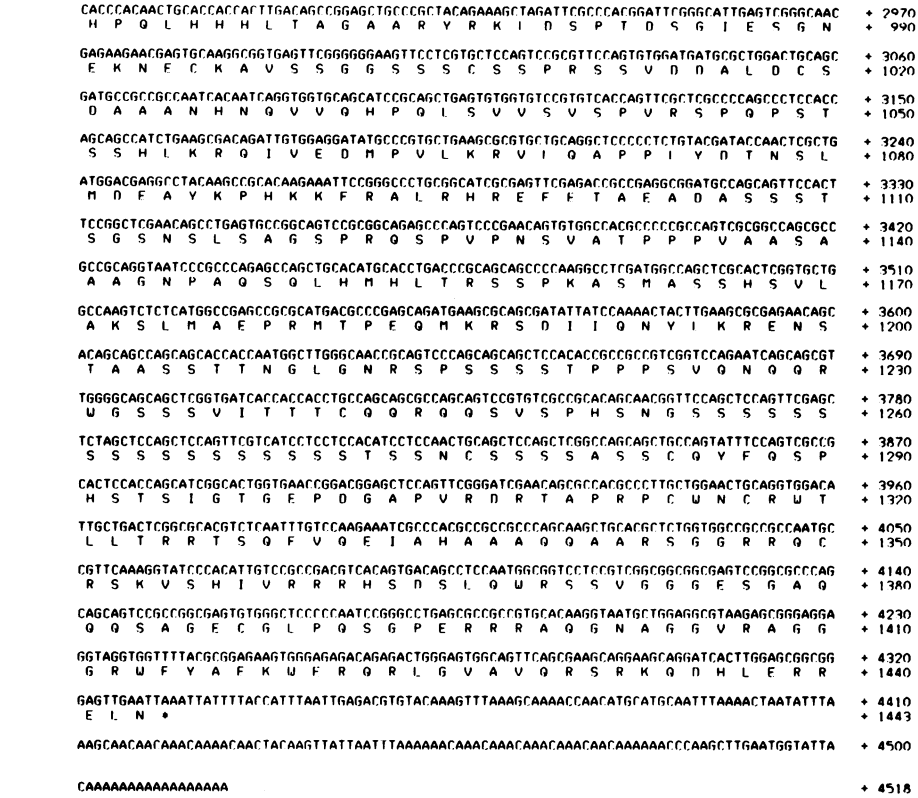


Fig. 2. cDNA sequence and predicted amino acid sequence of *75B* protein. Nucleotides are numbered in the 5′−3′ direction, beginning with the first ATG triplet encoding the putative methionine initiation amino acid. The nucleotides on the 5′-side of residue 1 are indicated by negative numbers. The number of the nucleotide residue at the right end of each line is given. The deduced *75B* amino acid sequence is shown below the nucleotide sequence. Amino acid residues are numbered beginning with the putative initiation methionine. Numbers of the last residue are given on the right-hand side. The termination codon TAA at the end of ORF is marked by an asterisk.

the chromosomal walk with respect to the centromere were delimited by *in situ* hybridization experiments. ^{3}H-labeled TOM11 DNA and ^{3}H-labeled TOM14 DNA either separately or together were hybridized to squash preparations of salivary gland polytene chromosomes of *Drosophila melanogaster* 3rd instar larvae. As shown in Fig. 1B, ^{3}H-labeled TOM14 DNA hybridized to the proximal end and ^{3}H-labeled TOM11 DNA to the distal end of the puff at 75B. The chromosomal walk encompassed the major part of the DNA in the puff at 75B. Exploratory Northern blot experiments were carried out by systematically hybridizing cloned 75B DNA to RNA extracted from salivary glands of 3rd instar larvae incubated for 2 to 6 h with or without 20-OH-ecdysone in tissue culture medium. A number of transcripts were detected and analyzed by characterizing corresponding cDNA (data not shown). With probes of TOM6 one transcription unit was detected, the expression of which was apparently regulated by 20-OH-ecdysone. TOM6 DNA is located in the proximal part of the 75B puff. Transcript(s) derived from this transcription unit have been designated 20-OH-ecdysone inducible *75B* transcripts.

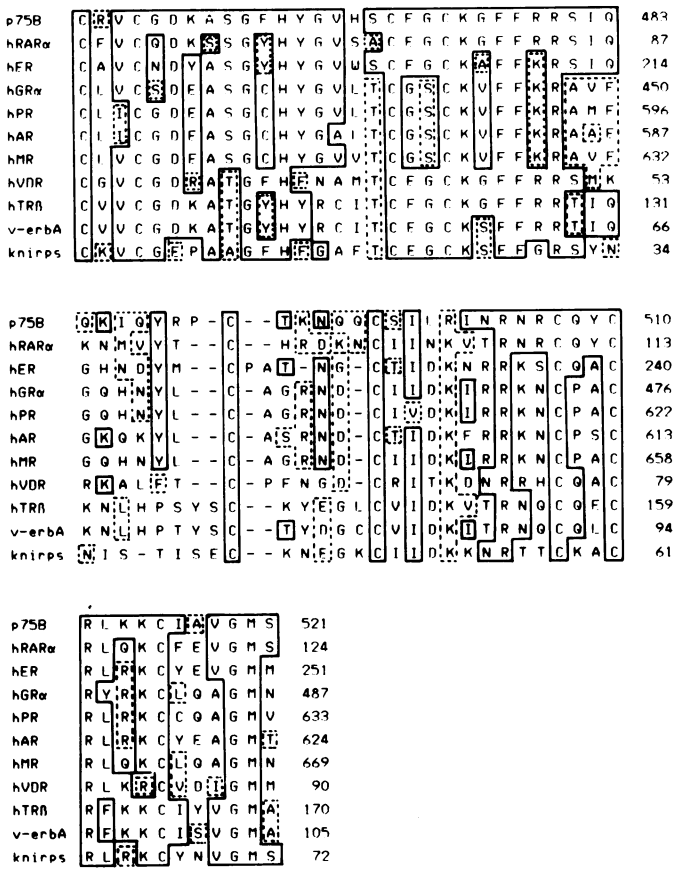


Fig. 3. Alignment of the 'zinc-finger' domain of *75B* protein with sequences of other 'zinc-finger' proteins, in particular with the corresponding domains of members of the steroid hormone and thyroid hormone receptor superfamily. hRARα = human retinoic acid receptor α [25]; hER = human estrogen receptor [33]; hGRα = human glucocorticoid receptor α [34]; hPR = human progesterone receptor [35]; hAR = human androgen receptor [36]; hMR = human mineralocorticoid receptor [37]; hVDR = human vitamin D receptor [38]; hTRβ = human thyroid hormone receptor β [39]; v-erbA = v-erbA oncogene product [26]; knirps = deduced protein sequence of *kni* gene [40]. Identical amino acids are boxed. Conservative amino acid substitutions are enclosed by a dashed line. Amino acids were grouped as follows: A,S,G,T; D,E,N,Q; F,Y,W; V,L,I,M; K,R; [27].

The longest cDNA clone, *c75B*, which was obtained by screening cDNA libraries with TOM6 DNA probes, was sequenced. The cDNA sequence is 4528 bp long (Fig. 2). The longest open reading frame derived from the cDNA sequence started at the 11th nucleotide. We did not succeed in isolating further cDNA molecules extending the 5′-sequence of the cDNA. Therefore, we cannot exclude at present that the *75B* cDNA open reading frame might be somewhat longer than the 4329 nucleotides shown in Fig. 2. The derived amino acid sequence of the *75B* protein is composed of 1443 amino acids with a calculated molecular weight of 156.2 kDa. The protein can be divided into three domains; an amino terminus (amino acids 1−453), a 'zinc finger' domain (amino acids 454−521) and a long carboxy terminus (amino acids 522−1443). A search in the NBFR data bank did not reveal

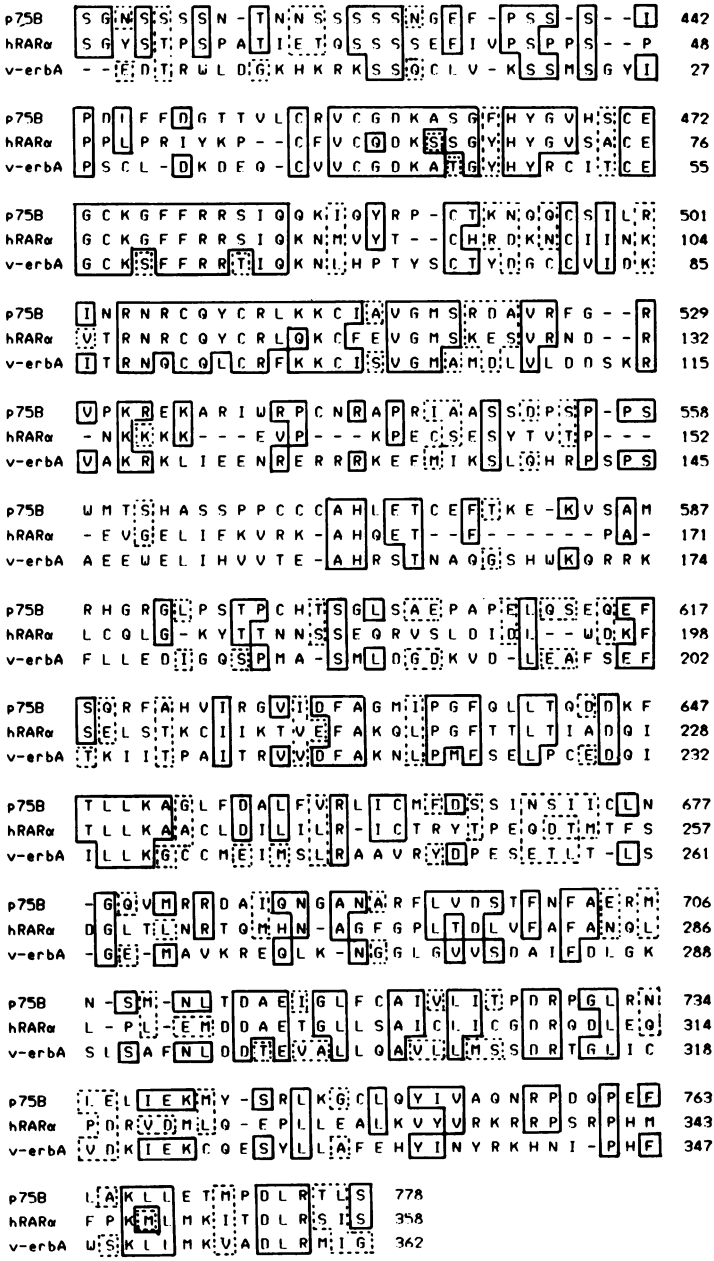


Fig. 4. Alignment of the 'zinc-finger' domain and the beginning of the amino terminus of *75B* protein with sequences of the human retinoic acid receptor [25] and of v-erbA protein [26]. Identical amino acids are boxed. Conservative amino acid substitutions are enclosed by a dashed line. Amino acids were grouped as in Fig. 3.

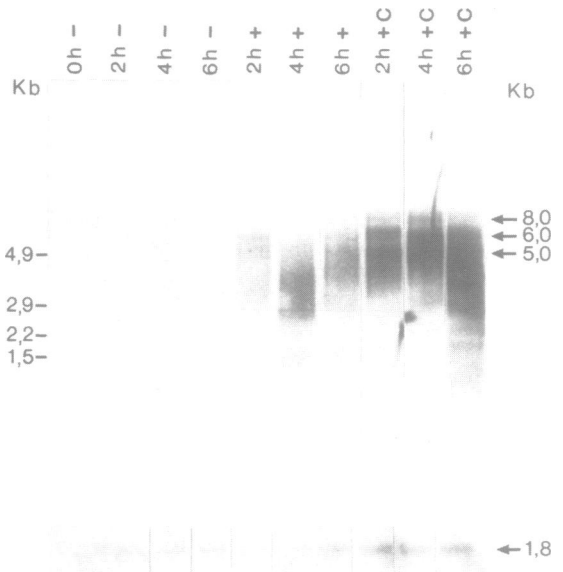


Fig. 5. Induction of *75B* transcripts by 20-OH-ecdysone. A ^{32}P-labeled cDNA probe was used for Northern hybridization. Size markers in kb are shown on the left side. To check the amounts and the integrity of RNA Northern blots were reprobed with probes to β1-tubulin [28]. The results are shown under each corresponding lane. The size of β1-tubulin mRNA is 1.8 kb. RNA was from PS1 salivary glands of 3rd instar larvae incubated *in vitro* for the indicated time span without (−), with 5×10^{-6}M 20-OH-ecdysone (+) and with 5×10^{-6}M 20-OH-ecdysone together with 1×10^{-4}M cycloheximide (+C).

any significant similarity with the amino terminus of the *75B* protein. However, the middle domain, which is rich in cysteines and basic amino acids shows striking similarities to the corresponding sequences of the steroid and thyroid hormone receptor superfamily (Fig. 3) [21,22,23]. These conserved sequences have the structure characteristic for the DNA-binding 'zinc-finger' motif of the *Xenopus* 5S rRNA transcription factor TFIIIA [24]. The 'zinc-finger' domain of the *Drosophila 75B* protein has the highest similarity with the ones of the human retinoic acid receptor [25] and of the v-erbA protein (thyroid hormone receptor) [26], respectively. The alignment of the derived *75B* and retinoic acid receptor protein sequences (Fig. 3) shows that of the 68 amino acids belonging to the 'zinc-finger' domain, 43 are identical. If one takes conservative amino acid substitutions into account [27], then 80% of the sequence is conserved between the domains of the *Drosophila 75B* and the human retinoic acid receptor proteins. Part of the carboxy terminus of the *75B* protein is also similar to the ones of the human retinoic acid receptor and of the v-erbA protein. If one allows for conservative amino acid substitutions, 44% of the first 257 amino acids of the *75B* carboxyterminal domain have been conserved with respect to the corresponding sequence of human retinoic acid receptor (Fig. 4). These sequence comparisons suggest that the *75B* protein is a member of the steroid and thyroid hormone receptor superfamily.

Expression of 75B transcripts

The induction of *75B* transcripts in 3rd instar larvae by 20-OH-ecdysone was investigated by studying *in vitro* the effect of 20-OH-ecdysone on *75B* mRNA expression. PS1 salivary

glands [3] were dissected from 3rd instar larvae and were explanted in tissue culture medium. Incubations were carried out from 2 to 6 h at 25°C in the presence of 5×10^{-6}M 20-OH-ecdysone. Control incubations were carried out either without added 20-OH-ecdysone or with 10^{-4}M cycloheximide added together with 20-OH-ecdysone. Subsequently, *75B* mRNA expression was analyzed by Northern blot experiments with total RNA isolated from the salivary glands (Fig. 5). Two major transcripts, approximately 5.0 and 6.0 kb in size, were detected with the *75B* cDNA probe. Incubations of 4h apparently induced larger quantities of *75B* transcripts than shorter or longer incubations as indicated by a comparison with the control hybridizations with β1-tubulin cDNA [28]. Also, incubations of salivary glands with 20-OH-ecdysone in the presence of cycloheximide lead to an accumulation of *75B* transcripts. In this case, an additional *75B* transcript (8kb) was detected, which is only faintly visible in 20-OH-ecdysone incubations (compare lane 5 with lane 8 in Fig. 5).

Ecdysteroid receptor has been detected in many different tissues of 3rd instar larvae of *Drosophila melanogaster* [8], e.g. salivary glands, imaginal discs and fat body. Therefore, 20-OH-ecdysone has apparently many different target tissues in 3rd instar larvae. Although it has been reported that 20-OH-ecdysone induces puffs at 75B on polytene chromosomes of salivary glands as well as of fat body [29], it is not clear whether the induction always leads to accumulation of *75B* mRNA in the different target tissues of 20-OH-ecdysone. The tissue distribution of *75B* RNA was investigated by *in situ* hybridizations to serial sagittal tissue sections of 3rd instar larvae. The sections were hybridized with an antisense-RNA probe derived from *75B* cDNA.

The results show that *75B* mRNA was detected in tissues like fat bodies, salivary glands and imaginal discs, but most remarkably, the strongest signal was seen in larval brain (Fig. 6). These observations demonstrate that *75B* mRNA is present in many tissues of 3rd instar larvae. It suggests that 20-OH-ecdysone possibly induces in many (if not all) larval target tissues the accumulation of *75B* mRNA(s). The translation product, therefore, should play a central role in mediating the regulation of the larval molt by 20-OH-ecdysone.

DISCUSSION

20-OH-ecdysone induces two prominent puffs on polytene chromosomes of 3rd instar larvae of *Drosophila melanogaster*, at loci 74EF and 75B. Previously, we have characterized an apparently 20-OH-ecdysone regulated gene in puff 74EF encoding two alternative transcripts [9]. The expression of one transcript decreased upon incubation of salivary glands with 20-OH-ecdysone *in vitro*. The alternative transcript increased in response to 20-OH-ecdysone and its expression correlated with the 20-OH-ecdysone induced activity of puff 74EF. The two *74E* transcripts are translated into two different proteins which have alternative amino terminal ends. It was suggested that the *74E* proteins function as alternative transcription factors and that the activity of the 20-OH-ecdysone inducible puff 74EF leads to a transcription factor switch.

The structure of the 20-OH-ecdysone inducible gene product in puff 75B suggests that this other prominent ecdysteroid regulated early puff also encodes a protein which is possibly involved in the regulation of transcription. In contrast to *74E* mRNA, however, *75B* mRNA was not detected in Northern blot experiments, if the RNA was extracted from PS1 salivary glands, which had not been incubated with 20-OH-ecdysone (Fig. 5). The appearance of *75B* mRNA required the incubation of PS1 salivary glands with 20-OH-ecdysone. As the

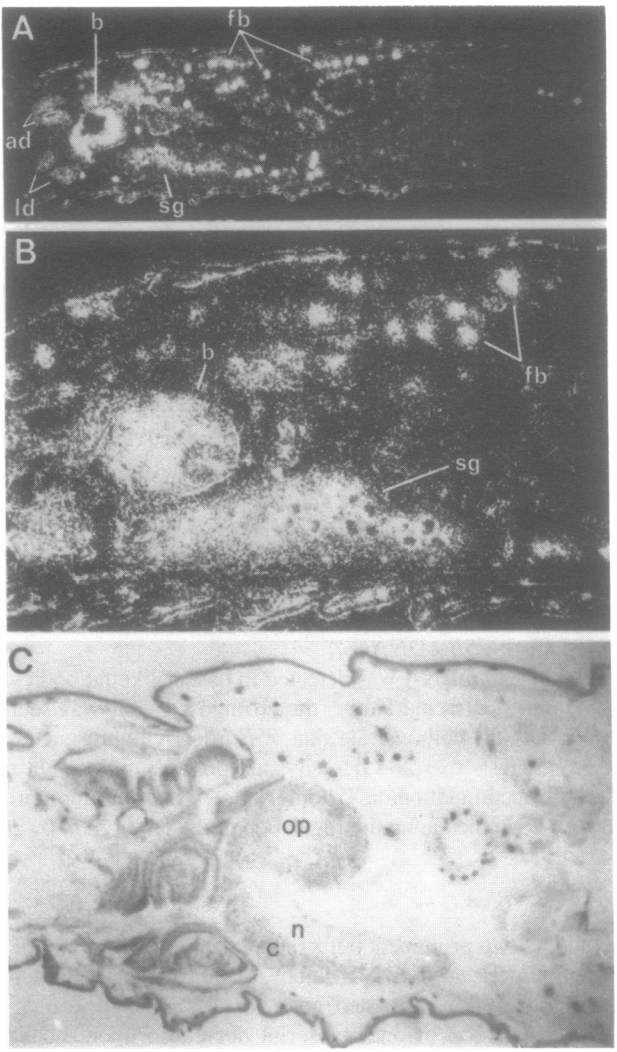


Fig. 6. In situ hybridization of a riboprobe from *c75B* DNA to tissue sections. (A) Dark field view of a third instar larva in sagittal section. Anterior is to the left and dorsal is up. ld = leg disc, ad = antenna disc, b = brain, s = salivary gland, fb = fat body. (B) Sagittal section of 3rd instar larva at higher magnification. Anterior is to the left and dorsal is up. b = brain, s = salivary gland, fb = fat body. (C) Bright field view of a third larval instar CNS in saggital section. Anterior is to the left and dorsal is up. n = neuropile, c = cortex, op = developing optic ganglia.

75B mRNA appearance and its insensitivity towards the presence of cycloheximide in the incubation medium parallels the behaviour of the 75B puff, the *75B* mRNAs encode apparently 20-OH-ecdysone inducible product(s). We do not know yet whether the *75B* mRNAs encode alternative reading frames like in the case of *74E* proteins or differ in their non-translated sequences. In any case, the derived open reading frame for the *75B*

mRNA(s) indicates that the *75B* protein has a striking similarity with members of the steroid and thyroid hormone receptor superfamily. Members of this family have a common design. They have a highly variable amino terminus (in length and in sequence), a conserved middle part of 66–68 amino acids and a less conserved carboxy terminus, which is also variable in length [21,22,23]. The derived *75B* protein sequence exhibits a similar design. The middle part, resembling a 'zinc-finger' motif, is highly conserved and the carboxy terminus is similar in part of its sequence with carboxy termini of other members of this hormone receptor family. The highest sequence similarities were observed between the *75B* protein and the human retinoic acid receptor. Therefore, it is quite possible that the *75B* protein binds a similar ligand. Juvenile hormone and retinoic acid are related structures. However, it is still a matter of conjecture whether juvenile hormone, retinoic acid or a derivative thereof is the ligand which is specifically bound by *75B* protein. This hypothesis is presently tested with fusion proteins. It is well known that steroid and thyroid hormones and retinoids have important functions in the mammalian nervous system [30]. In this context, it should be noted that the *in situ* hybridizations revealed a prominent expression of *75B* mRNA in larval brain indicating that two members of the receptor superfamily, *75B* protein as well as apparently ecdysteroid receptor, function in the insect nervous system.

Incubation of PS1 salivary glands with 20-OH-ecdysone leads to a rapid and transient induction of early puffs, most notably at 2B5, 74EF, and 75B. Studies with aneuploids, which were either duplicate (three doses) or deficient (one dose) for the 74EF and 75B early puffs, showed that early puffs compensate by their activity for altered gene doses [6]. In one dose genotypes these puffs are active for a longer time and in three dose genotypes for a shorter time than in the usual two dose genotype. These data are best understood if the activities of puffs 74EF and 75B are controlled by the concentration of their own gene products in a feedback type mechanism. Such feedback type mechanisms have frequently been observed, when the gene products—like possibly the *74E* and *75B* proteins—are transcription factors [31]. Also, many transcription factors interact synergistically with steroid receptors [32]. The *74E* and *75B* proteins might similarly interact with each other as well as with ecdysteroid receptor. Such a multifactorial network, similar to a recently proposed model [8], could readily account for the sequential and temporal gene activities exerted by 20-OH-ecdysone. With the gene products in hand, these predictions are now testable.

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