The EMBO Journal vol.6 no.12 pp.3729–3734, 1987

An ecdysone response element in the *Drosophila* hsp27 promoter

Guy Riddihough and Hugh R.B.Pelham

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Communicated by H.Pelham

It has previously been shown that a region of ~100 bp in the *Drosophila* hsp27 promoter is sufficient to confer ecdysone inducibility on a heterologous gene. We now show, using binding and DNase I footprinting assays, that a 23-bp hyphenated dyad within this sequence forms a protein-binding site, and that this is sufficient for inducibility. The sequence shows partial homology with mammalian steroid receptor binding sites. UV crosslinking identifies an 80- to 90-kd protein that binds specifically to this sequence and is thus a candidate for the ecdysone receptor.

Key words: ecdysone/*Drosophila*/hsp27/transcription

Introduction

The regulation of transcription by steroid hormones has been extensively studied in mammalian and chick cells (for review see Yamamoto, 1985). The steroids are bound by a receptor protein which, in the absence of hormone, is present in a cytoplasmic complex with the heat shock protein hsp90. Upon hormone binding, the receptor dissociates from hsp90, moves to the nucleus and binds to specific DNA sequences near hormone-activated genes (Redeuilh *et al.*, 1987; Sanchez *et al.*, 1987 and references therein). The receptor then activates transcription of those genes by a mechanism which is presumably analogous to that of other enhancer-binding proteins. cDNAs encoding the glucocorticoid and oestrogen receptors have been isolated (Hollenberg *et al.*, 1985; Green *et al.*, 1986; Miesfeld *et al.*, 1986). These proteins have homologous amino acid sequences, and recognize distinct but related DNA binding sites. The optimal binding sites have dyad symmetry, and are ~15 bp long (Beato, 1986; Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Jantzen *et al.*, 1987).

By comparison, the ecdysone response in *Drosophila* is less well understood. The mechanism is likely to be similar to that of other steroid response systems, but the receptor has not been well characterized and attempts to define functional promoter elements (its presumed binding sites) have failed to identify any reliable consensus sequence or any single element that is sufficient for hormone induction. The most extensively studied promoters have been those of the four small heat shock genes of *Drosophila*, which are known to be activated by ecdysone as well as by heat shock (Ireland *et al.*, 1982; Morganelli *et al.*, 1985). We have previously identified a region of ~100 bp from one of these genes, hsp27, which confers ecdysone inducibility on a heterologous promoter (Riddihough and Pelham, 1986). We show here that a dyad-symmetric sequence within this region is sufficient for inducibility, and is bound by a protein of ~90 kd that is a good candidate for the ecdysone receptor. The sequence shows similarities with the glucocorticoid and oestrogen response elements, but is longer.

Results

Identification of a protein-binding site

We used a gel retardation assay to search for proteins in *Drosophila* tissue-culture cells that can bind to the region of the hsp27 promoter that is responsible for ecdysone induction. The appropriately labelled DNA fragment was incubated with increasing amounts of a nuclear extract from Schneider 1 cells in in the presence of poly[d(I–C)] carrier, and the mixture electrophoresed on an acrylamide gel. Figure 1a shows that a discrete DNA–protein complex could be detected with this probe when moderate amounts of nuclear extract were assayed; at higher protein concentrations, all the probe accumulated in a complex or aggregate which barely entered the gel. Similar amounts of this complex were obtained with extracts from ecdysone-treated and untreated cells (Figure 1a), and addition of ecdysone to the binding reaction had no quantitative or qualitative effect (Figure 1b). The specificity of the binding was tested by the addition of plasmid DNA containing the probe sequences in a pUC vector, or vector alone. As shown in Figure 1c, the complex migrating at the top of the gel was not observed when low levels of either competitor were present. The lower labelled band was significantly reduced in intensity by 100 ng of the specific competitor, whereas a 10-fold higher concentration of the non-specific competitor was required to produce the same effect. This level of non-specific competitor represents ~1000-fold weight excess over the labelled probe. We conclude from this experiment that the protein which forms the more rapidly migrating complex binds to the hsp27 sequences in a specific manner, although it does have a detectable affinity for sequences in pUC DNA.

To identify the region of the DNA fragment that was bound by protein, we performed DNase I footprinting experiments with the same DNA fragment and cell extracts. Protection of a single region of ~23 bp was observed whichever DNA strand was labelled (Figure 2). Examination of the corresponding DNA sequence revealed a hyphenated dyad that was almost precisely covered by the footprint (Figure 2).

The amount of extract required to produce a footprint was roughly equivalent to the amount required to retard the mobility of all the probe in the gel assay (compare Figures 1 and 2), although precise correlation is difficult because of the non-linearity of the latter assay. In order to confirm that both assays detect the same DNA–protein complex, we prepared a synthetic double-stranded oligonucleotide corresponding to the protected dyad sequence and added it to a binding assay containing the longer labelled fragment. As shown in Figure 3, 35 ng of unlabelled oligonucleotide was sufficient to abolish formation of the specific labelled complex. In contrast, the amount of probe retained at the top of the gel was relatively unaffected by the presence of the oligonucleotide, even at a 10-fold higher concentration. This material, which is sensitive to the presence of pUC DNA (Figure 1b), evidently results from interaction of the

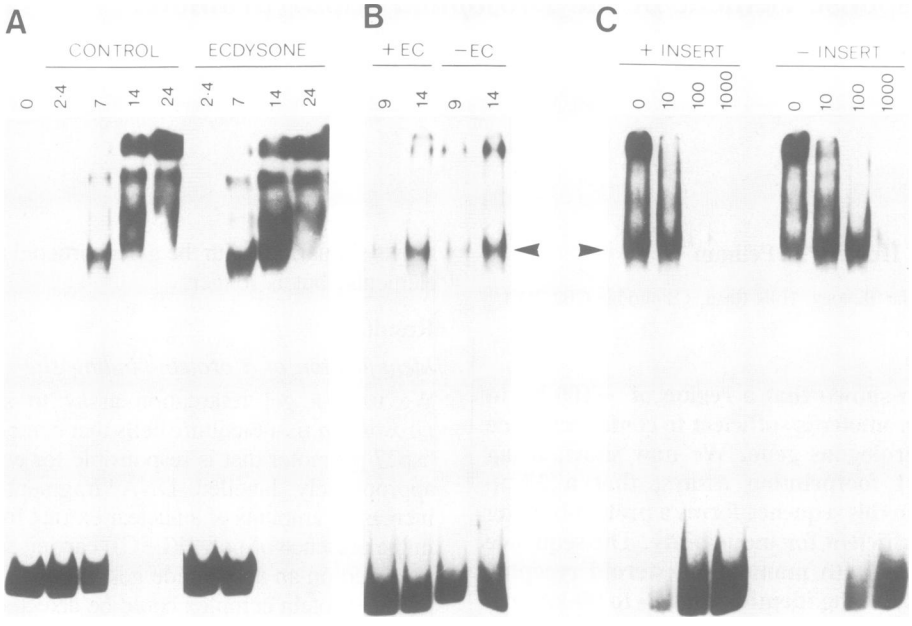

Fig. 1. Detection of protein−DNA complexes. A labelled probe consisting of the −579 to −455 region of the hsp27 promoter was mixed with varying amounts of nuclear extract and subjected to gel electrophoresis. Free DNA is visible at the bottom of the gels; arrowheads indicate the specific complex. **A**, assay of extracts from control and ecdysone-treated cells. The amount of extract protein (μg) is indicated at the top of each lane. **B**, extract from ecdysone-treated cells (9 or 14 μg) was assayed in the presence (+EC) or absence (−EC) of 10 μM ecdysone. **C**, extract (14 μg) was assayed with increasing amounts of *Msp*I-cut pUC plasmid containing (+ insert) or lacking (− insert) the probe sequences. The amount of competitor DNA added (ng) is indicated at the top of each lane.

probe with relatively non-specific DNA-binding proteins.

Taken together, the gel retardation and DNase footprinting experiments identify a single specific protein-binding site within the ecdysone-responsive region. This sequence thus seems a good candidate for the hormone receptor-binding site.

The binding site confers ecdysone inducibility

Previous deletion analysis has shown that removal of the binding site defined above from the complete hsp27 promoter reduces but does not eliminate ecdysone induction *in vivo* (Riddihough and Pelham, 1986). to investigate whether the site itself was sufficient for inducibility, we inserted the synthetic oligonucleotide (corresponding to bases −553 to −527 of the hsp27 promoter) upstream of the TATA box of a truncated hsp70 promoter, which in turn was fused to the chloramphenicol acetyl transferase (CAT) gene. This construct was introduced into *Drosophila* tissue-culture cells, and its response to hormone treatment determined by CAT assay. As a control for the efficiency of transfection and hormone treatment, its behaviour was compared with that of the plasmid pCatX1, which has the entire hsp27 promoter fused to the CAT gene. Figure 4 shows the results of a typical experiment. In the absence of hormone treatment both pCatX1 and the oligonucleotide construct yielded very little activity. With ecdysone treatment the construct containing the synthetic binding site was strongly induced, whereas an equivalent plasmid lacking the binding site was not. This experiment was performed three times, each with duplicate transfections. In each case the synthetic construct had no activity above background in the absence of hormone; after hormone treatment the activity varied from 57 to 70% of that produced by pCATX1. These results show that the synthetic dyad is sufficient to confer ecdysone inducibility on a heterologous promoter.

The experiment shown in Figure 4 also included three constructs in which larger fragments of the hsp27 promoter were fused to the hsp70 TATA box. The activity of these plasmids has been described previously; none produce any activity in the absence of hormone (Riddihough and Pelham, 1986). The −540/−484 construct includes only the right-hand half of the dyad binding site and is inactive. Inclusion of the complete dyad restores activity, and it is increased still further when additional 3′ sequences are present. It is not clear whether this elevated activity is due to a more favourable location of the dyad relative to the TATA box, or to the presence of other protein binding sites that have not yet been detected *in vitro*. We favour the latter explanation, because even when the −553/−527 region is disrupted, the hsp27 promoter still shows some ecdysone inducibility, implying the existence of other response elements (Riddihough and Pelham, 1986).

UV-induced crosslinking of protein to the ecdysone regulatory element

To identify the polypeptide that binds to the ecdysone response element, we used UV-induced crosslinking of a ^{32}P-labelled, BUdR-substituted DNA probe to the binding protein. This technique has been used successfully to identify a number of DNA-binding proteins. Preliminary experiments confirmed that a BUdR-substituted probe derived from a cloned version of the synthetic dyad was indistinguishable in gel retardation assays from the normal thymidine-containing probe. A binding reaction was then subjected to UV irradiation and the crosslinked DNA−protein complexes analysed by electrophoresis on an SDS-containing gel. The specificity of the crosslinking was checked by the addition of unlabelled competitor oligonucleotides to the binding reaction. Figure 5 shows the results of such experiments. Several labelled bands are apparent, none of which were reduced in intensity by the addition of an unrelated oligonucleotide (a heat shock regulatory sequence; Figure 5a, HSE). Most were reduced only slightly by the addition of up to 500 ng of the synthetic dyad oligonucleotide (Figure 5a, ECD). These probably represent proteins of limited specificity which contribute to the material at the

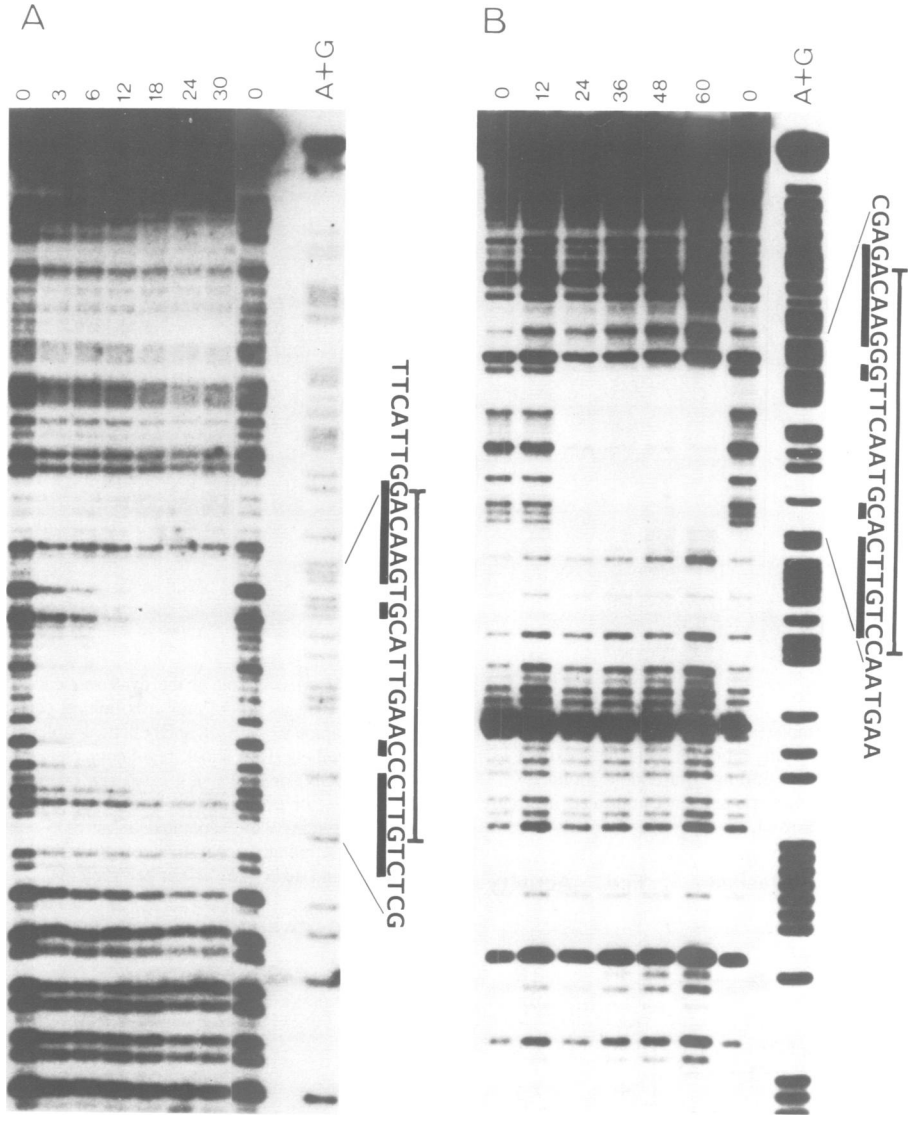

Fig. 2. Footprinting of the ecdysone response element. The probe used in Figure 1 was labelled at the 3′ end of the lower (**A**) or upper (**B**) strand, mixed with nuclear extract from ecdysone-treated cells and digested with DNase I. The amount of extract (μg) is indicated above the lanes. The relevant sequence is shown for each strand; the thin bar indicates the protected area, and thick bars the hyphenated dyad symmetry in this region.

top of the gel in a normal binding assay. The crosslinking experiments were performed with relatively high concentrations of protein (20 μg) and complexes that are poorly competed by the oligonucleotide are abundant under these conditions (see Figure 3).

Only one band was eliminated by the addition of unlabelled competitor at a level that was sufficient to abolish specific binding of labelled probe in the gel retardation assay (Figure 5). The mobility of the band suggests that the protein has a mol. wt of ~90 kd. This is an upper estimate, because of the presence of at least a portion of the labelled DNA on the protein, but experience suggests that DNA fragments of this size do not have a very significant effect on protein mobility in this gel system (Sorger and Pelham, 1987). As found for the binding activity in the previous assays, this protein was detected whether or not the cells were treated with ecdysone (Figure 5b). It is likely, however, that the method used to preare the protein extracts would activate a steroid receptor even in the absence of hormone (see Discussion). We thus conclude from the DNA-binding experiments that the best candidate for the ecdysone receptor is a polypeptide with a mol. wt of 80–90 kd.

Other ecdysone-inducible genes

Since the other small heat shock genes of *Drosophila* also respond to ecdysone, we examined their promoter sequences for elements related to the binding site defined above. The sequences searched in hsp22, hsp23 and hsp26 are known to be necessary and sufficient for ecdysone induction of these genes (Morganelli *et al.*, 1985; Klemenz and Gehring, 1986; Mestril *et al.*, 1986). However, although some weak homologies could be detected, they were no more common in these genes than in others that do not respond to ecdysone.

As a more direct test, a portion of the hsp22 promoter region that is known to be sufficient for hormone induction was subjected to DNase I footprint analysis using crude nuclear extract. Protection of heat shock regulatory elements and sequences of the form CTCTCTCT were observed, but no footprints that were competable with the hsp27 binding site could be detected (data

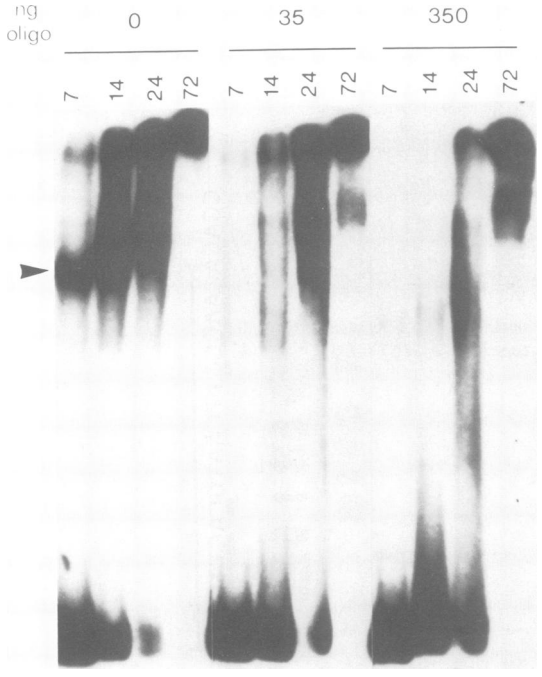

Fig. 3. A synthetic oligonucleotide completes for complex formation. Binding assays were performed with varying amounts of nuclear extract as in Figure 1, with the addition of 0, 35 or 350 ng of a double-stranded oligonucleotide comprising the dyad symmetric sequence indicated in Figure 2. The specific complex is indicated (arrow).

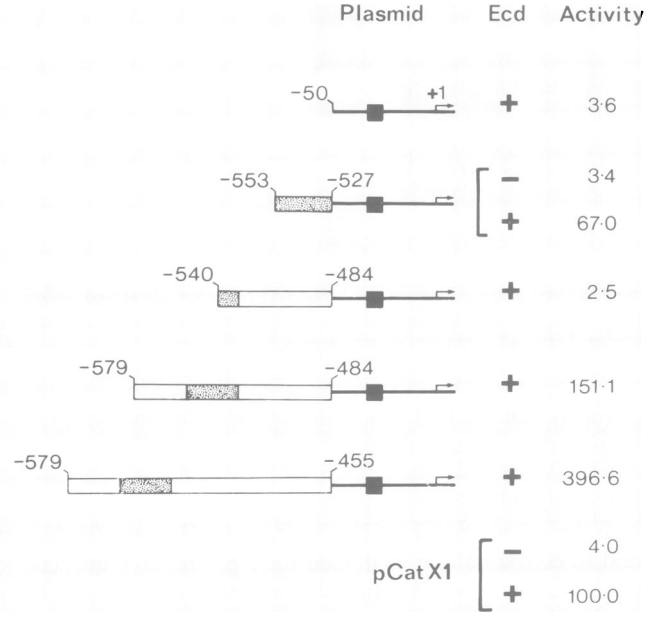

Fig. 4. Activity of promoter elements *in vivo*. The synthetic dyad or larger fragments of the hsp27 promoter were inserted upstream of the TATA box of an hsp70-CAT fusion gene, the plasmids transfected into *Drosophila* tissue-culture cells, and CAT activity determined without (−) or with (+) ecdysone treatment of the cells. Activities are expressed as a percentage of that obtained with pCatX1, which contains the entire hsp27 promoter fused to the CAT gene. The plasmid structures are shown schematically, the black box representing the hsp70 TATA sequence, and the stippled box the dyad sequence. The first plasmid is a control with no regulatory sequences.

not shown). Thus, if there are binding sites for the same protein in the hsp22 ecdysone-responsive region, they are too weak to be easily detected in this way. Attempts to find oligonucleotide-competable complexes using gel retardation of a variety of probes from the hsp22, hsp23 and hsp26 genes were also unsuccessful. However, these experiments were complicated by the presence of other protein-binding sites in these promoters, and by the high levels of non-specific binding seen with crude extracts. It is thus unlikely that we would have detected binding sites that are substantially weaker than the hsp27 one.

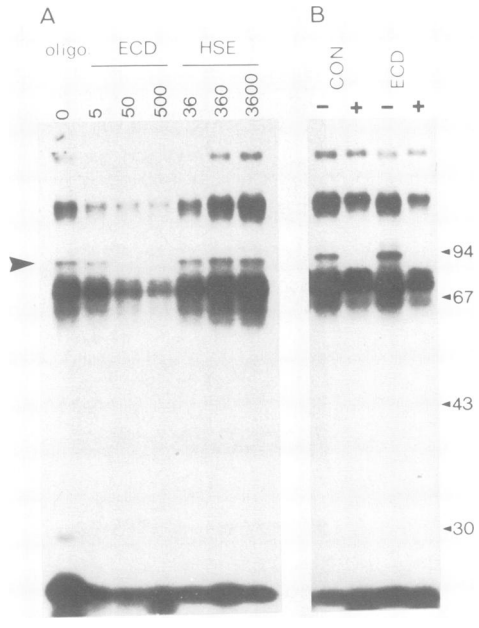

Fig. 5. UV crosslinking of the dyad sequences to proteins. **A**, the synthetic dyad was used as probe, and crosslinking performed in the presence of the indicated amounts (ng) of unlabelled, double-stranded synthetic dyad (ECD) or of a synthetic heat shock regulatory element (HSE). **B**, extracts of control (CON) or ecdysone-treated (ECD) cells were crosslinked in the absence (−) or presence (+) of 200 ng of unlabelled synthetic dyad. The labelled proteins were separated on an SDS-containing gel. The large arrow indicates the putative ecdysone receptor; the numbers at the right show the positions and sizes (kd) of marker proteins.

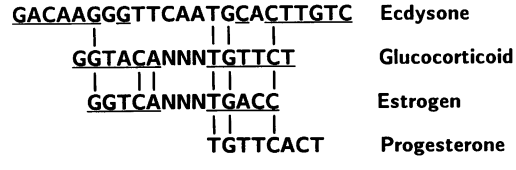

Fig. 6. Comparison of the hsp27 ecdysone response element with the consensus binding sites for the glucocorticoid, oestrogen and progesterone receptors.

Discussion

The results presented here identify a 23-bp hyphenated dyad in the *Drosophila* hsp27 promoter, which confers ecdysone inducibility on a heterologous gene. The position of this sequence correlates quite well with our previous deletion analysis: in the complete promoter, a 5′ deletion that removes the left half of the dyad significantly reduces ecdysone inducibility, and a further deletion that removes the entire dyad has an even stronger effect. The persistence of a weak hormone inducibility suggests, however, that other response elements also exist (Riddihough and Pelham, 1986).

Figure 6 shows a comparison of the dyad element with the consensus binding sites for the glucocorticoid, oestrogen and pro-

gesterone receptors (Bailly *et al.*, 1986; Beato, 1986; Klein-Hitpass *et al.*, 1986; Seiler-Tuyns *et al.*, 1986; Jantzen *et al.*, 1987). Like the ecdysone element, the glucocorticoid and oestrogen sites have dyad symmetry, probably reflecting the dimeric nature of the receptors, with an odd number of bases at the centre of the dyad. The progesterone consensus sequence suggested by Bailly *et al.* (1986) corresponds to one-half of the other sites—such 'half sites' are also seen in regions footprinted by the glucocorticoid receptor (Yamamoto, 1985). Strikingly, three bases are conserved in all three of the vertebrate steroid elements, and are also found in the right half of the ecdysone element. This homology suggests a common mode of interaction between protein and DNA for all of the receptors, and supports the idea that they are evolutionarily related. Systematic mutageneis could provide a better definition of the optimal ecdysone response element, and reveal whether the homologous bases are important for protein binding.

It is suprising that we have not been able to identify ecdysone response elements in the promoters of the other small heat shock genes, even though we know from previous work that their 5′ flanking sequences are sufficient for hormone inducibility in tissue-culture cells. In particular, little homology to the hsp27 sequence can be seen in the small regions of the hsp23 and hsp22 promoters that have been implicated in hormone induction (Mestril *et al.*, 1986; Klemenz and Gehring, 1986). Even in the hsp27 gene there appear to be sequences 3′ to the dyad which increase activity *in vivo*, but to which we have not observed any binding of proteins *in vitro*. It may be that these genes contain multiple sites which show little similarity to each other and bind the receptor only weakly, but which in combination can produce a substantial response. This could explain why it has proved difficult to identify short sequences that are sufficient for induction in genes other than hsp27 (e.g. Mestril *et al.*, 1986); the element we have found may be a rare example of a strong receptor binding site.

We have tentatively identified the ecdysone receptor as a polypeptide of 80−90 kd, based on UV-induced crosslinking of this protein to the response element and the assumption that the latter is directly recognized by the receptor, as in other systems. There is no evidence that the presence of ecdysone is required for specific interaction of this protein with DNA, but this is not inconsistent with results from other systems. The glucocorticoid and progesterone receptors can bind DNA in the absence of hormone (Bailly *et al.*, 1986; Sanchez *et al.*, 1987), and mutants of the glucocorticoid receptor have been constructed which lack the steroid-binding domain but still show specific interaction with DNA and activation of transcription (Hollenberg *et al.*, 1987; Miesfeld *et al.*, 1987; Rusconi and Yamamoto, 1987). It seems reasonable to suggest that the main role of the hormone is to dissociate the receptor from hsp90 (hsp83 in *Drosophila*), thus freeing it for binding to DNA (Groyer *et al.*, 1987; Sanchez *et al.*, 1987). The conditions used to prepare *Drosophila* cell extracts, which involve exposure to 0.4 M NaCl, may be sufficiently harsh to disrupt the putative receptor−hsp83 complex without the need for hormone—it is known that ammonium sulphate can activate the DNA-binding properties of the glucocorticoid receptor in the absence of hormone (Sanchez *et al.*, 1987).

Materials and methods

Oligonucleotides, plasmids and probes

Synthetic oligonucleotides CTAGACAAGGGTTCAATGCACTTGTCCATCG and CGATGGACAAGTGCATTGAACCCTTGT were annealed and used directly as competitor or cloned into the *Xba*I−*Hin*cII sites of pUC18. UV-crosslinking probe was prepared from this plasmid by primer extension (in the direction *Eco*RI to *Hin*dIII) on collapsed supercoiled templates with [^{32}P]dATP and BrdUTP (Chen and Seeburg, 1985), followed by excision of the insert with *Eco*RI and *Hin*dIII. For bandshift and footprinting probes, a fragment of the hsp27 promoter extending from a *Bal*31-derived endpoint at −579 to a *Dra*I site at −455 (see Riddihough and Pelham, 1986) was inserted between the *Bam*HI and *Hin*cII sites of pUC18. The probes were labelled by filling in the *Eco*RI or *Hin*dIII site as appropriate, and cut with *Pst*I or *Sac*I respectively. The HSE competitor used in Figure 5A consisted of double-stranded, ligated copies of the sequence CTAGAAGCTT (Sorger and Pelham, 1987).

The CAT fusion genes depicted in Figure 4 have mostly been described previously (Riddihough and Pelham, 1986). The synthetic dyad was cloned into the same position as the promoter fragments, between a polylinker-derived *Xba*I site and the *Nru*I site at −50 of the hsp70 promoter. In the control plasmid the *Xba*I site was filled in and fused directly to the *Nru*I site.

Binding assays

Nuclear extracts of Schneider 1 cells were prepared as described by Wu (1985). To avoid nuclease activity, the binding reactions were performed in the absence of Mg^{2+}. A typical reaction contained, in 20 μl, 2 μg poly[d(I−C)], 2mM EDTA, 1 mM spermidine−HCl, 1 ng probe DNA, and up to 3 μl of nuclear extract. The volume was made up with solution III: 20 mM HEPES pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol, 1 mM PMSF, 0.5 mM TPCK, 25 μM TLCK, 2 μg/ml Pepstatin A. Binding, gel electrophoresis, DNase I footprinting and UV crosslinking were performed as described by Sorger and Pelham (1987). All binding reactions were for 15 min at 30°C. DNase I footprinting was performed with a 40-μl binding reaction, but the protein amounts indicated in Figure 2 are those used per standard 20-μl reaction, to aid comparison with other figures. For UV crosslinking, the reaction contained 20 μg of protein, and was diluted 5-fold with solution III before UV irradiation for 2 min. Protein was separated from excess probe by phenol extraction followed by ethanol precipitation of the phenol phase with 2.5 volumes of ethanol. Except where indicated in Figures 1A and 5B, all reactions were performed with extracts from cells treated for 24 h with 2 μM ecdysone.

CAT assays

Transfection of Schneider 1 cells, treatment with ecdysone, harvesting and CAT assays were performed as described previously (Riddihough and Pelham, 1986). To ensure a linear response of the assay, quantities of cell extract which resulted in <20% acetylation of the chloramphenicol were used. In the experiment shown in Figure 4, the induced activity of pCatX1 corresponded to 4.9% conversion of the chloramphenicol to the acetylated form.

Acknowledgements

We thank Peter Sorger, Mike Lewis and Richard Treisman for helpful technical advice, and Terry Smith for the synthetic oligonucleotides.

References

Bailly,A., Le Page,C., Rauch,M. and Milgrom,E. (1986) *EMBO J.*, **5**, 3235−3241.

Beato,M. (1986) In Puett,D., Ahmad,F., Block,S., Lopez,D.M., Melner,M.H., Scott,W.A. and Whelan,W.J. (eds), *Advances in Gene Technology: Molecular Biology of the Endocrine System.* Cambridge University Press, Cambridge, pp. 250−254.

Chen,E.Y. and Seeburg,P. (1985) *DNA*, **4**, 165−170.

Green,S., Walter,P., Kumar,V., Krust,A., Bornert,J.-M., Argos,P. and Chambon,P. (1986) *Nature*, **320**, 134−139.

Groyer,A., Schweizer-Groyer,G., Cadepond,F., Mariller,M. and Baulieu, E.-E. (1987) *Nature*, **328**, 624−626.

Hollenberg,S.M., Weinberger,C., Ong,E.S., Cerelli,G., Oro,A., Lebo,R., Thompson,E.B., Rosenfield,M.G. and Evans,R.M. (1985) *Nature*, **318**, 635−641.

Hollenberg,S.M., Giguere,V., Segui,P. and Evans,R.M. (1987) *Cell*, **49**, 39−46.

Ireland,R.C., Berger,E., Sirotkin,K., Yund,M.A., Osterbur,D. and Fristrom,J. (1982) *Dev. Biol.*, **93**, 498−507.

Jantzen,H.-M., Strahle,U., Gloss,B., Stewart,F., Schmid,W., Boshart,M., Miksicek,R. and Schutz,G. (1987) *Cell*, **49**, 29−38.

Klein-Hitpass,L., Schorpp,M., Wagner,U. and Ryffel,G.U. (1986) *Cell*, **46**, 1053−1061.

Klemenz,R. and Gehring,W.J. (1986) *Mol. Cell. Biol.*, **6**, 2011−2019.

Mestril,R., Schiller,P., Amin,J., Klapper,H., Ananthan,J. and Voellmy,R. (1986) *EMBO J.*, **5**, 1667−1673.

Miesfeld,R., Rusconi,S., Godowski,P.J., Maler,B.A., Okret,S., Wikstrom, A.-C., Gustafsson,J.-A. and Yamamoto,K.R. (1986) *Cell*, **46**, 389−399.

Miesfield,R., Godowski,P.J., Maler,B.A. and Yamamoto,K.R. (1987) *Science*, **236**, 423−427.

Morganelli,C.M., Berger,E.M. and Pelham,H.R.B. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5865–5869.
Redeuilh,G., Moncharmont,B., Secco,C. and Baulieu,E.-E. (1987) *J. Biol. Chem.*, **262**, 6969–6975.
Riddihough,G. and Pelham,H.R.B. (1986) *EMBO J.*, **5**, 1653–1658.
Rusconi,S. and Yamamoto,K.R. (1987) *EMBO J.*, **6**, 1309–1315.
Sanchez,E.R., Meshinchi,S., Tienrungroj,W., Schlesinger,M.J., Toft,D.O. and Pratt,W.B. (1987) *J. Biol. Chem.*, **262**, 6986–6991.
Seiler-Tuyns,A., Walker,P., Martinez,E., Merillat,A.-M., Givel,F. and Wahli,W. (1986) *Nucleic Acids Res.*, **14**, 8755–8770.
Sorger,P.K. and Pelham,H.R.B (1987) *EMBO J.*, **6**, 3035–3041.
Wu,C. (1985) *Nature*, **317**, 84–87.
Yamamoto,K.R. (1985) *Annu. Rev. Genet.*, **19**, 209–252.

Received on August 20, 1987; revised on September 8, 1987