The steroid hormone 20-hydroxyecdysone is a key regulatory factor, controlling blood-meal triggered egg maturation in mosquitoes. To elucidate the ecdysone hierarchy governing this event, we cloned and characterized the ecdysone receptor homologue, from the mosquito, Aedes aegypti, which form a functional complex capable of ligand and DNA binding. Here we analyzed the DNA-binding properties of the AaEcR:AaUSP heterodimer with respect to the effects of nucleotide sequence, orientation, and spacing between half-sites in natural Drosophila and synthetic ecdysone response element (EcREs). By using an electrophoretic gel mobility shift assay, we showed that AaEcR:AaUSP exhibits a broad binding specificity, forming complexes with inverted (IR) and direct (DR) repeats of the nuclear receptor response element half-site consensus sequence AGGTCA separated by spacers of variable length. A single nucleotide spacer was optimal for both imperfect (IR<sup>hsp</sup>-1) and perfect (IR<sup>p</sup>-1) inverted repeats; adding or removing 1 base pair in an IR<sup>hsp</sup>-1 spacer practically abolished binding. However, changing the half-site to the consensus sequence AGGTCA (IR<sup>p</sup>-1) increased binding of AaEcR:AaUSP 10-fold over IR<sup>hsp</sup>-1 and, at the same time, reduced the stringency of the spacer length requirement, with IR<sup>p</sup>-0 to IR<sup>p</sup>-5 showing detectable binding. Spacer length was less important in DRs of AGGTCA (DR-0 to DR-5); although 4 bp was optimal, DR-3 and DR-5 bound AaEcR:AaUSP almost as efficiently as DR-4. Furthermore, AaEcR:AaUSP also bound DRs separated by 11–13 nucleotide spacers. Competition experiments and direct estimation of binding affinity (K<sub>D</sub>) indicated that, given identical consensus half-sites and an optimal spacer, the AaEcR:AaUSP heterodimer bound an IR with higher affinity than a DR. Co-transfection assays utilizing CV-1 cells demonstrated that the mosquito EcR:USP heterodimer is capable of transactivating reporter constructs containing either IR-1 or DR-4. The levels of transactivation are correlated with the respective binding affinities of the response elements (IR<sup>p</sup>-1 > DR-4 > IR<sup>hsp</sup>-1). Taken together, these analyses predict broad variability in the EcREs of mosquito ecdysone-responsive genes.

Nuclear hormone receptors activate or repress transcription through direct association with specific sequences known as hormone response elements (HREs), in the regulatory regions of responsive genes (1–5). Known HREs contain characteristic 6-base pair core sequences, found either singly or as half-sites within inverted, direct, or everted repeats. The specificity of an HRE is derived from four important characteristics as follows: the nucleotide sequence of each half-site, the spacing between half-sites, half-site orientation, and composition of the flanking regions (4, 6). Steroid hormone receptors, including the estrogen (ER), progesterone, glucocorticoid, and mineralocorticoid receptors, were originally thought to bind exclusively as homodimers to inverted repeats (IR) with the consensus half-site AGGTCA or AGAACA separated by three nucleotides (IR-3) (3, 6). However, glucocorticoid and estrogen receptors have recently been shown to form complexes on direct repeats with variable spacing between half-sites (7).

The vitamin D receptor (VDR) and non-steroid nuclear receptors, including thyroid hormone (TR) and retinoic acid (RAR) receptors, bind to cognate response elements as heterodimers with a shared partner, the retinoid X receptor (RXR) (4). Response elements for these receptors are composed of direct repeats with consensus half-sites, AGGTCA, spaced by 3, 4, or 5 nucleotides (DR-3, DR-4, and DR-5) (8). The subsequent demonstration that a DR-1 serves as an RXR and peroxisome proliferator activating receptor response element, and that a DR-2 serves as a second RAR response element has expanded the model to the so-called 1 to 5 rule (9). More recently, widely spaced, directly repeated AGGTCA elements have been shown to act as promiscuous enhancers for different classes of nuclear receptors; for example, a DR-15 reporter gene can be activated by RAR-RXR, VDR-RXR, and ER (10). These heterodimers can also regulate target gene expression by binding to response elements consisting of inverted or everted repeats. For example, an inverted repeat without spacing (IR-0) has been reported to function as a response element for TR, RAR, and VDR, whereas inverted repeats with a 6-bp spacer or a 12-bp spacer serve as response elements for TR and VDR, respectively (11–13).

The insect steroid hormone, 20-hydroxyecdysone (20E), regulates essential processes in development, molting, metamorphosis, and reproduction (14–18). The functional ecdysone re-
Mosquito EcR-USP DNA Binding and Transactivation

cceptor in Drosophila is a heterodimer of the edysone receptor (EcR) protein (19) and an RXR homologue, Ultraspireacle (USP) (20–22). The first edysone response element (EcRE) was identified in the promoter of the Drosophila heat shock protein-27 gene. It is an imperfect palindromic with only a 1-bp spacer (IR²⁻¹), rather than the 3-bp spacer typical of vertebrate steroid HREs (23). Several EcREs have been identified in the regulatory regions of the following four more Drosophila genes: Eip28/29 (24), Fbp-1 (25), Sgs-4 (26), and Lsp-2 (27), each containing imperfect inverted repeats with a 1-bp spacer (IR-1). These findings, together with DNA binding and in vitro transactivation studies, suggested that natural Drosophila EcREs are predominantly IR-1s. However, it was later found that Drosophila EcR-USP (DmEcR-DmUSP) can bind synthetic DRs of (A/G)GGTCA with spacers of 3–5 nucleotides and can activate reporter gene constructs containing these direct repeats in Drosophila Schneider-2 (S2) cells (28, 29). Finally, the EcRE of the Drosophila nested gene (ng) is a direct repeat of AGGTCA with a 12-nucleotide spacer (30).

The maintenance and dispersal of mosquito-borne disease depends upon successful reproduction of the mosquito, and 20E plays a crucial role in regulation of vitellogenesis and oogenesis (15, 16, 31, 32). The processes of egg maturation and disease transmission are intimately associated through the mutual requirement for blood. Therefore, elucidation of the role of edysone receptor in mosquito reproduction is of significant biological and epidemiological importance. Although several target genes for the 20E-mediated regulatory cascade have been identified (32–34), native EcREs in mosquitoes are still unknown. In the mosquito Aedes aegypti, cDNAs of one edysone receptor (AaEcR) (35) and two USP isoforms (36) have been cloned. Compared with vertebrate nuclear receptors, insect EcR and USP homologues show unexpectedly high levels of sequence diversity (36, 37). It is, therefore, difficult to predict the DNA binding specificity of the mosquito EcR-USP heterodimer. Although DNA binding domain determinants of half-site sequence specificity have been identified (38–40), determinants of half-site spacing and orientation as well as flanking sequence preferences are less well understood. In order to address these questions, we have analyzed the DNA binding properties of the AaEcR-AaUSP heterodimer. We used electrophoretic gel mobility shift assays (EMSA) with synthetic oligonucleotides and in vitro synthesized AaEcR and AaUSP to investigate the effects of the sequence, orientation, and spacing of half-sites on the DNA binding properties of the mosquito EcR-USP heterodimer. Finally, we have used CV-1 cells in order to correlate the DNA binding properties of AaEcR-AaUSP with their ability to transactivate the reporter gene constructs containing EcREs.

MATERIALS AND METHODS

In Vitro Synthesis of Nuclear Receptor Proteins—The nuclear receptor proteins were synthesized by coupled in vitro transcription-translation (TNT) system (Promega). AaEcR and AaUSP cDNAs containing full open reading frames were subcloned into pGEM3Z (Promega) as described previously (36). For comparison, the 2.1-kilobase pair EcoRI fragment from pG7-1-DmUSP (41) and the 3.3-kilobase pair BamHI fragment from pACT-DmEcR-B1 (19) bearing the entire open reading frames of Drosophila USP and EcR, respectively, were subcloned into pGEM7 (+) (Promega). The in vitro transcription/translation reactions programmed by the circular plasmid DNAs utilized the SP6 promoter. To confirm the synthesis of proteins with expected sizes, control TNT reactions were performed in the presence of 35S-methionine, and the resulting reactions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Oligonucleotides and Probes—Oligonucleotides were purchased either from the Macromolecular Structure Facility of the Biochemistry Department at Michigan State University or Life Technologies, Inc. For DNA binding studies, a pair of sense and antisense oligonucleotides was annealed and resolved by 15 or 20% non-denaturing PAGE, and the appropriate bands of double-stranded oligonucleotides were electroeluted. Ten picomoles of double-stranded oligonucleotides were end-labeled with T4 DNA kinase (Life Technologies, Inc.) and 50 µCi of [γ³²P]ATP (NEN Life Science Products), and the unincorporated radioactive activity was removed through a Sephadex G-25 (Amerham Pharmac Biotech) spin column.

Electrophoretic Gel Mobility Shift Assay (EMSA)—One microtiter of each TNT reaction was used alone or in combination as a protein source for EMSA. Proteins were incubated for 30 min at room temperature in 20 µl of the electrophoretic mobility shift buffer, containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 µg/ml poly(dI-dC)-poly(dI-dC), 0.32 mg/ml single-stranded DNA (5'-CTAAACAGTCGCTGACTGAAGCCGGCC-3'), 0.5 µM 20E, and for competition experiments, the indicated amounts of unlabeled competitor oligonucleotides. This was followed by the addition of 0.05 pmol of [³²P]-labeled probe and incubation for another 30 min. The reaction mixture was resolved using a 6% non-denaturing PAGE at a constant voltage of 150 V for 90 min at room temperature. The gel was dried, and the distribution of radioactivity was visualized either by autoradiography or by PhosphorImaging for quantitative analysis using ImageQuant™ software (Molecular Dynamics).

Equilibrium Dissociation Constant (Kₐ) Estimation—Kₐ values of AaEcR-AaUSP binding to potential EcREs were estimated according to the EMSA procedure (42) using the EMSA procedure described above. Protein samples were first incubated in the electrophoretic mobility shift buffer containing 0.5 µM 20E for 30 min and then with several different concentrations of labeled double-stranded oligonucleotides for another 30 min. Bound and free probes were separated by non-denaturing PAGE and quantified by the PhosphorImager. Saturation curves and Scatchard plots (43) were calculated for at least three independent experiments, and the mean value was taken as the Kₐ.

Antibodies—The antiserum raised against AaEcR (anti-AaEcR) was prepared as follows. The HincII-EcoRI fragment of the AaEcR cDNA clone was subcloned into pMAL-c2 (New England Biolabs) and expressed in Escherichia coli TB1 strain. The AaEcR protein, which was fused to maltose-binding protein, was concentrated by amyllose resin according to the manufacturer’s instructions. The fusion protein was further purified by SDS-PAGE followed by electrodialysis. A New Zealand White rabbit was immunized by subcutaneous injection with 100 µg of the purified fusion protein emulsified in TiterMax (CytRx) adjuvant. Blood was collected at 2-week intervals, and the titer of specific antibodies was estimated by Western blotting of the TNT reaction programmed by pGEM3Z-AaEcR.

Monoclonal antibody against DmUSP (anti-DmUSP), described in Khoury Christianson et al. (44), was a gift from Dr. F. C. Kafatos (European Molecular Biological Laboratories, Heidelberg, Germany).

Reporter and Expression Plasmids and Cell Transfection Assays—The BamH1-EcoRI fragment of AaEcR cDNA (35) and the EcoRI fragments of AaUSP cDNA (36) were subcloned into the corresponding sites of pCDNA3.1/neo (Invitrogen). Translatability of these constructs was ensured by in vitro TNT-coupled transcription/translation (Promega), which was followed by EMSA to verify binding properties of the expressed receptors. The reporter plasmid ΔMTV-5xIR²⁻¹-CAT (chloramphenicol acetyltransferase), containing five copies of IR²⁻¹ was used in initial transfection assays (22). To make other reporter plasmids, oligonucleotides IR²⁻¹-1 (agtctcggGGTTCaGTCACGTtgctgctcag), DR-4 (agctcggTGGAGTCGTCACGTtgctgctcag), and IR²⁺⁻¹ (agctcggGATTCAgTCACGTtgctgctcag) were ligated into the HindIII site of ΔMTV-CAT (45). Constructs harboring a single copy of either IR²⁻¹, IR²⁺⁻¹, or DR-4 were used for a comparative study of transactivation by EcR-USP. Three copies of DR-4 were placed before the CAT gene to make the reporter construct ΔMTV-3xDR4-CAT. All reporter constructs were confirmed by sequencing. The expression plasmid CMV-β-galactosidase was a kind gift from Dr. L. Karl Olson (Department of Physiology, Michigan State University).

The green African monkey kidney CV-1 cell line (American Tissue Culture Collection, Bethesda, MD) was maintained in Dulbecco’s modified Eagle’s medium containing 10% calf serum. 2 x 10⁶ cells were seeded in 6-well plates for 18–24 h before transfection. Transfection was performed using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. In brief, 0.4 µg each of AaEcR, AaUSP, and CMV-β-galactosidase expression plasmid, and 1.2 µg of the reporter plasmids were mixed with LipofectAMINE and transfected in OPTI-MEM (Life Technologies, Inc.) for 3–5 h. The transfection mixture was removed, and the cells were further incubated in OPTI-MEM supplemented by 5% charcoal-stripped calf serum for 36–48 h in the presence of ethanol vehicle or 1 µM Muristerone A (Sigma). Each well
received 2.4 μg of total DNA. pCDNA3.1/Zeo (+) was used as a carrier for equalizing the amount of DNA allocated to each well. CAT assays were performed as described by Herbomel et al. (46) for 2 h and β-galactosidase assays for 60–90 min. CAT activity was normalized with β-galactosidase activity.

RESULTS

Binding of the AaEcR-AaUSP Heterodimer to Inverted Repeats: the Effect of Spacer Length and Half-site Nucleotide Sequence—By using EMSA with in vitro TNT-expressed mosquito EcR and USP isomorphs, we previously demonstrated that the AaEcR-AaUSP complex bound a 30-base pair oligonucleotide corresponding to the Drosophila hsp27 EcRE (36). This element was designated as IR\textsuperscript{hsp}-1 (an imperfect inverted repeat with a 1-bp spacer). In this study, we report in detail the DNA binding characteristics of mosquito EcR heterodimerized with the mosquito USP\textsubscript{b} isomorph (designated hereafter as USP). Our analyses indicated, however, that the binding properties of the AaEcR-AaUSP\textsubscript{a} heterodimer are generally similar to those of AaEcR-AaUSP\textsubscript{b} (not shown).

First, we confirmed binding of the AaEcR-AaUSP heterodimer to IR\textsuperscript{hsp}-1 by utilizing anti-AaEcR and anti-DmUSP antibodies: when EMSA were performed in the presence of either of the antibodies, AaEcR-AaUSP-IR\textsuperscript{hsp}-1 complexes were supershifted (not shown). Next, we investigated the role of IR spacer nucleotide length in the binding of the AaEcR-AaUSP heterodimer. In the first series of experiments, we tested the ability of IR\textsuperscript{hsp}s with various spacer lengths to compete against IR\textsuperscript{hsp}-1 binding to the AaEcR-AaUSP complex (Fig. 1). A 50-fold molar excess of the appropriate cold IR was added to each EMSA reaction with radiolabeled IR\textsuperscript{hsp}-1, and the intensity of the resulting bands was measured. Self-competition with cold IR\textsuperscript{hsp}-1 led to a 96% reduction in binding intensity (Fig. 1, A and B). IR\textsuperscript{hsp}-0 also was revealed to be an efficient competitor, displacing 82% of the bound IR\textsuperscript{hsp}-1 probe. However, the ability of IR\textsuperscript{hsp}s to compete with IR\textsuperscript{hsp}-1 progressively declined as the number of spacer nucleotides increased from 2 to 5. In a second series of experiments, the direct binding of IR\textsuperscript{hsp}-0–5 to AaEcR-AaUSP was tested by EMSA: apart from IR\textsuperscript{hsp}-1, only IR\textsuperscript{hsp}-0 exhibited a detectable retarded band (not shown).

Next, we tested AaEcR-AaUSP binding to a perfect inverted repeat consisting of AGGCTCA half-sites with a 1-bp spacer (IR\textsuperscript{pers}-1). The EMSA revealed a strong retarded band when both TNT-generated AaEcR and AaUSP proteins were included in the reaction (Fig. 2A, lane 1). Binding of AaEcR-AaUSP to IR\textsuperscript{pers}-1 was confirmed by supershift assays incorporating anti-AaEcR and anti-DmUSP (Fig. 2A, lanes 2 and 3). Interestingly, a faster migrating weak band, which was supershifted by anti-DmUSP, was detected when AaUSP alone was tested with IR\textsuperscript{pers}-1 (not shown).

Perfect inverted repeats with nucleotide spaces from 0 to 5 (IR\textsuperscript{pers}-0–5) had much higher affinity to AaEcR-AaUSP than the imperfect repeat motifs (IR\textsuperscript{hsp}-0–5); the retarded bands were clearly recognizable in all reactions (Fig. 2B). The retarded bands with IR\textsuperscript{pers}-0 and IR\textsuperscript{pers}-1 were considerably more intense than those formed by IR\textsuperscript{hsp}-2 to IR\textsuperscript{hsp}-5. Together with the results of IR\textsuperscript{hsp}s, this indicates preferential binding of AaEcR-AaUSP to IR motifs with 0 and 1 nucleotide spacers (Fig. 2B). This preference was also confirmed by competition EMSA (Fig. 3). AaEcR-AaUSP was incubated with a fixed amount of radiolabeled IR\textsuperscript{pers}-1 and competed by increasing amounts of unlabeled IR\textsuperscript{pers}s of various spacers. In the self-competition control, binding was almost completely competed away by inclusion of a 25-fold molar excess of cold IR\textsuperscript{pers}-1. IR\textsuperscript{pers}-0 also competed well, with a 25-fold molar excess competing away 93% of IR\textsuperscript{pers}-1 binding to AaEcR-AaUSP. Twenty-five-fold molar excess of IR\textsuperscript{pers}-2, IR\textsuperscript{pers}-3, and IR\textsuperscript{pers}-5 displaced only 56, 11, and 32% of binding, respectively. IR\textsuperscript{pers}-5 consistently showed stronger competition than IR\textsuperscript{pers}-3. Efficiency of competition indicates that the DNA binding affinity of AaEcR-AaUSP toward IR\textsuperscript{pers}s follows the order IR-1 > IR-0 > IR-2 > IR-5 > IR-3.

Binding of the AaEcR-AaUSP Heterodimer to Direct Repeats of AGGCTCA—We also measured the binding of mosquito EcR-USP to a set of synthetic elements containing direct re-
peaks with spacers ranging from 0 to 5. The mosquito EcR-USP complex effectively bound direct repeats (DRs) of AGGTCA containing a 4-bp spacer (DR-4) (Fig. 4). The composition of the AaEcR-AaUSP complex was verified by supershift experiments using either anti-AaEcR or anti-DmUSP. The latter super-

shifted the AaEcR-AaUSP-DR-4 complex as efficiently as it did the control complex DmEcR-DmUSP-DR-4 (not shown).

We investigated the possible effect of DR-4 flanking regions on AaEcR-AaUSP binding by testing the following three DR-4 response elements: DR-4/3C with flanking regions from Drosophila ng elements (30), DR-4/HS with flanking regions from Drosophila hsp27 EcRE (23), and DR-4/VT with flanking re-
gions from the thyroid response element (47). Radiolabeled IRhsp-1 was displaced from AaEcR-AaUSP equally efficiently by 50-fold molar excess of cold DR-4s containing any of the three flanking regions (Fig. 4, lanes 1–5). Labeled DR-4s with differ-
ent flanking regions strongly bound AaEcR-AaUSP, forming specific retardation bands of similar size and intensity (Fig. 4, lanes 6–11). Thus, the flanking DNA sequences did not much affect specific binding of the heterodimer. Also of note is that incubation of AaEcR-AaUSP with DR-4/3C resulted in an additional band of higher mobility than the specific heterodimer band (Fig. 4, lane 6). This high mobility band was competed by an excess of the cold specific probe (Fig. 4, lane 7) and was specifically supershifted with anti-DmUSP, but not anti-AaEcR antibodies (not shown), suggesting that it might repre-
sent the binding of AaUSP alone.

The role of the spacer nucleotides in DRs was also investi-
gated. In EMSA competition experiments, the AaEcR-AaUSP complex was incubated with labeled DR-4 in the absence of unlabeled competitor or in the presence of different molar excess of unlabeled competitor oligonucleotides IRm=0–5 (see Fig. 2 for sequences). Reactions were subjected to EMSA, and the radioactivity in the specific protein-DNA complexes was counted by PhosphorImaging. The radioactivity associated with the DNA-protein complex observed without competition was taken as the control and defined as 100%. Data are reported as a percentage of the control.

Fig. 3. Effect of spacer length of the perfect inverted repeats (IRm=5s) on binding with AaEcR-AaUSP. AaEcR-AaUSP was incubated with 0.05 pmol of 32P-labeled IRm=1 in the absence of unlabeled competitor or in the presence of different molar excess of unlabeled competitor oligonucleotides IRm=0–5 (see Fig. 2 for sequences). Reactions were subjected to EMSA, and the radioactivity in the specific protein-DNA complexes was counted by PhosphorImaging. The radioactivity associated with the DNA-protein complex observed without competition was taken as the control and defined as 100%. Data are reported as a percentage of the control.

Fig. 2. AaEcR-AaUSP bound to the perfect inverted repeats, IRm=5 sequences. A, EMSAs were done with AaEcR-AaUSP and an IRm=1 probe. The specifically retarded AaEcR-AaUSP-IRm=1 complex is indicated by an arrowhead (lane 1). The complex was supershifted by anti-AaEcR (lane 2, an arrow with open head) and anti-DmUSP (lane 3, an arrow with solid head) and could be competed away by a 50-fold molar excess of the unlabeled IRm=1 (lane 4). The position of unbound IRm=1 is indicated by an asterisk. B, direct binding EMSA assay. EMSAs were carried out with AaEcR-AaUSP and 0.05 pmol each of radiolabeled IRm=5s with 0 to 5 spacer nucleotides. Position of the shifted AaEcR-AaUSP-DNA complex is indicated by an arrowhead and of the free probe by an asterisk. Oligonucleotide probes used are as follows: IRm=0, agagaacaagAGGTCAATGACCTtgtccaa; IRm=1, agagaacaagAGGTCAATGACCTtgtccaa; IRm=2, agagaacaagAGGTCAATGACCTtgtccaa; IRm=3, agagaacaagAGGTCAATGACCTtgtccaa; IRm=5, agagaacaagAGGTCAATGACCTtgtccaa.
the mosquito EcR-USP complexes than in IRs. Nevertheless, efficiency of competition (Fig. 5) indicates that the DNA binding affinity of AaEcR:AaUSP toward DRs follows the order DR-4 > DR-3 > DR-5 > DR-2 > DR-1 > DR-0.

We were also interested in determining whether the mosquito EcR-USP complex might be capable of recognizing more widely spaced direct repeats. It has been reported that the Drosophila EcR-USP complex recognizes a DR-12 sequence found within the Drosophila ang gene (the original element was called DR-11 because 7 bp were taken as the consensus half-site) (30). The mosquito EcR-USP was capable of binding to the ang EcRE (Fig. 6); however, our analysis of this element identified more closely spaced cryptic direct repeats within the ang EcRE. Within the ang EcRE, there are consensus half-sites at either end and an imperfect half-site (AGGCCA) in the middle, such that it could form a DR-2 or DR-4 in combination with one of the terminal elements. Whereas binding of DmEcR:DmUSP was abolished when both terminal consensus half-sites in the DR-12 were mutated simultaneously (30), this does not rule out the possibility that the DR-2 or DR-4 might be the active element or might be a functionally significant part of a compound element.

To investigate the nature of AaEcR:AaUSP binding to the ang EcRE, we performed mutational analyses of all three half-sites in this sequence, in which a mutation was introduced independently into the 5′-proximal (DR-12/P), middle (DR-12/M), or 3′-distal (DR-12/D) half-sites. First, we conducted a competition assay with 32P-labeled IR 5′-1 and 5- or 25-fold molar excess of cold DR-12, DR-12/P, DR-12/M, or DR-12/D (Fig. 7). Twenty five-fold molar excess of cold DR-4, used as a positive control, removed 98% of radioactive probe binding (Fig. 7, lane 3), and 25-fold molar excess of the ang EcRE eliminated about 92% (Fig. 7, lane 5). Mutating the proximal half-site (DR-12/P) cripples the DR-12, leaving only the imperfect DR-4 intact. DR-12/P was much a weaker competitor (Fig. 7, lanes 6 and 7). In contrast, DR-12/M and DR-12/D, in which only the imperfect DR-2 was preserved, retained most of the binding ability of the original ang EcRE, with DR-12/M competing more strongly than DR-12/D (Fig. 7, lanes 8–11). The overall order of relative affinity of tested elements was DR-4 > ang EcRE = DR12/M > DR12/D >> DR12/P. Importantly, DR-12/M exhibited the same level of competition as the original DR-12, suggesting that the latter indeed serves as a response element with a 12-nucleotide spacer.

In order to determine whether other widely spaced direct repeats might also function as EcREs, we tested binding to
Sequence and Orientation of Half-sites in the Response Element—For these analyses, we utilized only IRhsp-1, IRper-1, and DR-4 (data not shown). EMSA analyses showed that AaEcR bound this composite motif as a heterodimer (Fig. 9), IR per-1, and DR-4 (not shown). The differences in DNA binding affinity, we calculated the equilibrium binding analyses and Scatchard analyses were used to estimate the half-site orientation and sequence on the DNA binding affinity of the AaEcR-AaUSP heterodimer. For these analyses, we utilized only IRhsp-1, IRper-1, and DR-4, because these elements exhibited maximal binding in the respective categories. First, we performed competitive EMSA, in which 32P-labeled IRhsp-1 was competed with 2.5-, 5.0-, and 10.0-fold molar excess of cold IRhsp-1, IRper-1, and DR-4/3C (lanes 1–3). The reactions were subjected to EMSA and autoradiographed. The AaEcR-AaUSP DNA complexes are indicated by an arrowhead and the free probe by an asterisk. Oligonucleotides used in this experiment are as follows: DR-4/HS, tggacaAGGTCAagAGTTCAgtgct; DR-12, aagcgaaAGGTCAagAGacataagAGGCCAaagaAGGTCAggaaaat; DR-12/P, aagcgaaAGacataagAGGCCAaagaAGGTCAggaaaat; DR-12/M, aagcgaaAGGTCAagAGacataagAGGCCAaagaAGGTCAggaaaat; DR-12/D, aagcgaaAGGTCAagAGGCCAaagaAGacatatagAGGCCAaagaAGGTCAggaaaat.

Binding Affinity of the AaEcR-AaUSP Heterodimer: Effect of Sequence and Orientation of Half-sites in the Response Element—We compared the effect of half-site orientation and sequence on the DNA binding affinity of the AaEcR-AaUSP heterodimer. For these analyses, we utilized only IRhsp-1, IRper-1, and DR-4, because these elements exhibited maximal binding in the respective categories. First, we performed competitive EMSA, in which 32P-labeled IRhsp-1 was competed with 2.5-, 5.0-, and 10.0-fold molar excess of cold IRhsp-1, IRper-1, and DR-4, or Eip28/29 (Fig. 8). The results suggest that the binding affinity of IRhsp-1 to AaEcR-AaUSP was stronger than that of DR-4 or IRper-1, which varied insignificantly from each other. The Eip28/29 appeared to have the weakest binding affinity to AaEcR-AaUSP. Finally, to resolve quantitatively the differences in DNA binding affinity, we calculated the equilibrium dissociation constants (Kd) for IRhsp-1, IRper-1, and DR-4 binding to AaEcR-AaUSP. The Ecr-USP heterodimer was incubated with increasing concentrations of radiolabeled probes (IRhsp-1, IRper-1, or DR-4) in the presence of 5 × 10^{-7} M 20E, the optimal concentration for Ecr-USP DNA binding.2 Saturation binding analyses and Scatchard analyses were used to estimate Kd values for the binding of AaEcR-AaUSP to IRhsp-1 (Fig. 9), IRper-1, and DR-4 (not shown). The differences in Kd values for IRhsp-1, IRper-1, and DR-4, which are in agreement with the results of the competition analyses (Fig. 8), indicate that AaEcR-AaUSP binds IRhsp-1 with an 8-fold higher affinity than to DR-4 and with 10-fold higher affinity than to IRper-1 (Table 1).

Transactivation of AaEcR-AaUSP: DNA Binding Affinity Corresponds to Transactivation Activity—Transactivation of AaEcR-AaUSP was studied using the CV-1 cell line. This mammalian cell line has no endogenous EcR and contains very low endogenous levels of RXR. It has been used to study transactivation of DmEcR-DmUSP (20–22). The transactivation ability of the AaEcR-AaUSP heterodimer was assessed with the ΔMTV-5xIRhsp-1-CAT reporter plasmid, which contains five tandem repeats of IRhsp-1. Transfection of CV-1 cells with the reporter plasmid alone resulted in a very low basal level of CAT activity (Fig. 10A). Co-transfection of the reporter plasmid with either AaEcR or AaUSP expression vector alone did not confer ecdysone responsiveness. However, strong induction (40-fold) of CAT activity was observed when the reporter plasmid was co-transfected with both AaEcR and AaUSP expression vectors and incubated with 1 μM MurA, demonstrating that the AaEcR-AaUSP heterodimer activated reporter gene expression in a ligand-dependent manner.

Next, we tested whether DR-4 could function as an EcRRE in CV-1 cells. We constructed a reporter plasmid (ΔMTV-3xDR4-CAT) containing three copies of DR4. Co-transfection of this reporter construct with either the AaEcR or the AaUSP expression vector did not render CV-1 cells ecdysone-responsive (data not shown). However, co-transfecting the reporter construct with both AaEcR and AaUSP expression vectors rendered CV-1 cells highly responsive to ligand with 9-fold induction (Fig. 10B).

Finally, we elucidated whether the level of transactivation by AaEcR-AaUSP depends on the sequence and orientation of the half-sites in the EcRRE and whether it is correlated with DNA binding affinity. We constructed ΔMTV-CAT reporter plasmids containing a single copy of IRhsp-1, DR-4, or IRper-1. Co-transfection of these reporter plasmids with either AaEcR or AaUSP expression vector did not render CV-1 cells ecdysone-
responsive similar to results with the ΔMTV-5xIRhsp-1-CAT reporter plasmid (data not shown). Co-transfection of both AaEcR and AaUSP with each of these reporter plasmids resulted in an increase in CAT activity in the presence of 1 mM MurA (Fig. 11). The level of transactivation of the reporter plasmid containing only one copy of the EcRE is considerably lower than that with five copies or three copies (Fig. 10). The differences in levels of activation between tested EcREs were of lower magnitude than the differences between their binding affinities. However, the strength of binding directly corresponds to the level of transactivation for each class of EcRE, with IRhsp-1, IRper-1, and DR-4 (Table I).

A

B

C

**FIG. 9.** Binding affinity ($K_d$) of IRhsp-1 to the AaEcR-AaUSP complex. A, EMSAs of AaEcR-AaUSP and radiolabeled IRhsp-1.

**TABLE I**

The equilibrium dissociation constants ($K_d$) of different DNA sequences binding to AaEcR-AaUSP and the corresponding level of reporter gene transactivation in CV-1 cells (means ± S.E.)

| EcRE   | $K_d$ (nM) | Induction |
|--------|------------|-----------|
| IRhsp-1 | 3.73 ± 0.85 | 2.29 ± 0.17 |
| IRper-1 | 0.326 ± 0.026 | 4.27 ± 0.38 |
| DR-4   | 2.21 ± 0.36  | 3.36 ± 0.38 |

a IRhsp-1, agagacaagGGTTCAaTGCACTtgtccaat.
b IRper-1, agagacaagAGGTCAaTGACCTtgtccaat.
c DR-4, aagcgaaAGGTCAaggaAGGTCAggaaaat.

In this paper, we provide further evidence that the AaEcR-AaUSP heterodimer is the functional mosquito ecdysone receptor and that it is capable of binding various DNA motifs oriented either as inverted or direct repeats. Data presented here parallels previous observations from several insect species that heterodimerization of EcR and USP is required for efficient binding of both the ligand and the response elements, as well as for gene transactivation (20–22, 36, 49, 50). Analyses utilizing EMSA and anti-EcR and anti-USP antibodies clearly demonstrated that the AaEcR-AaUSP heterodimer exhibits specific binding to the various sequences of naturally occurring *Drosophila* EcREs as well as to the synthetic response elements tested in this study. This and a previous study (36) demonstrate that the AaEcR-AaUSP heterodimer is capable of binding to various DNA motifs with the consensus half-site sequence AGGTCA oriented as inverted repeats. Indeed, EcREs found in native *Drosophila* genes are predominantly through the indicated range of DNA probe concentrations. Radioactivity associated with free oligonucleotides and with protein-oligonucleotide complexes was determined separately, permitting the construction of a saturation curve and a Scatchard plot (Fig. 9). EMSAs and quantifications were repeated at least three times.
shown that the perfect palindrome IRper-1 binds 10 times more strongly to the Eip28/29 and hsp27 EcREs (Fig. 8). Here, we have obtained similar results when testing mosquito ecdysone receptor binding sites and harvested for CAT assay. CAT activity was normalized by ß-galactosidase activity. Transfection was performed in triplicate and the normalized CAT activity averaged (mean ± S.E.).

Fig. 11. Comparison of MurA transcriptional induction conferred by IRUTm, DR-4, and IRUTm elements on ΔMTV-CAT reporter constructs. 0.4 µg each of CMV-β-galactosidase, AaEcR, and AaUSP expression vectors were transiently co-transfected with 1.2 µg of the ΔMTV-CAT reporter plasmid harboring one copy of IRUTm (column 1), DR-4 (column 2), and IRUTm (column 3). After transfection, cells were incubated in the presence of vehicle ethanol or 1 µM MurA for 36 or 48 h and harvested for CAT assays. Transfection was performed in two independent experiments in triplicate, and the CAT activity, normalized by β-galactosidase activity, was expressed as fold induction (mean ± S.E.). The differences in transactivation between the different elements are statistically significant (p < 0.01).

It has been demonstrated that Drosophila EcR-USP binds to direct repeats and that a 4-bp spacer is optimal (28, 29). Our observations on the mosquito EcR-USP heterodimer suggest that this aspect of EcR-USP DNA binding specificity also displays a high degree of functional conservation. Direct binding and competition assays demonstrate that the nucleotide spacer length is less important in direct repeats of AGGTCA (DR-0 to DR-5) than in IRs. Although 4 bp is an optimal spacer length in the direct repeats, DR-3 and DR-5 bind AaEcR-AaUSP almost as efficiently as DR-4. The order of binding affinities of AaEcR-AaUSP to DRs (DR-4 > DR-3 > DR-5 > DR-2 > DR-1 > DR-0) corresponds closely to that recently reported for DmEcRDmUSP (DR-4 > DR-5 > DR-3 > DR-1 > DR-2 > DR-0) (29).

Competition experiments and direct estimations of Kd indicate that binding affinity depends on the sequence of the half-site and is higher when the consensus is used for each half-site. However, given the same consensus half-site and an optimal spacer, the AaEcR-AaUSP heterodimer binds an inverted repeat with considerably more strength than a direct repeat. Our results significantly extend DNA binding studies on insect EcR-USP heterodimers by providing accurate measurements of dissociation constants for DR-4, IRUTm, and IRUTm.

Kato et al. (32) showed that in the chicken ovalbumin promoter region, there are multiple AGGTCA motifs arranged as direct repeats separated from each other by more than 100 bp. Despite such large spacers, they can act synergistically as a complex estrogen response element, indicating that widely spaced half-sites can cooperate to generate an efficient estrogen response element. Moreover, widely spaced direct repeats (10–200 bp) can function as cis-acting response elements for retinoic acid and vitamin D receptors (10). In contrast to the specificity observed with shorter spaced DRs (DR-1 to DR-5), different receptors bind promiscuously to these widely spaced inverted imperfect palindromes; the binding affinities of Sgs-4, Lsp-2, Fbp-D, and Eip28/29 EcREs are weaker than the hsp27 EcRE (IRUsp-1), which is the most efficient natural EcRE identified for Drosophila to date (23, 24, 26, 27, 51). We obtained similar results when testing mosquito ecdysone receptor binding to the Eip28/29 and hsp27 EcREs (Fig. 8). Here, we have shown that the perfect palindrome IRUsp-1 binds 10 times more efficiently than IRUsp-1.

Our results suggest that spacer length likewise plays an important role in both imperfect (IRUsp) and perfect (IRUsp) inverted repeats, with a single nucleotide spacer being optimal for both. This finding is in agreement with conclusions drawn from studies performed with DmEcRDmUSP (23, 24, 51). Moreover, we also found that whereas adding or removing one base pair from a spacer in IRUsp-1 practically abolishes binding, changing the half-site to the consensus sequence AGGTCA (IRUsp-1) reduces the stringency of the spacer length requirement, so that IRUsp-0 to IRUsp-5 exhibits detectable binding.
repeats to activate transcription in the presence of retinoic acid, vitamin D, or estrogen. Our tests have shown that although the AaEcR-AaUSP heterodimer exhibits relatively strong binding to DR-12, it is considerably weaker than to DR-3 or DR-4. Furthermore, AaEcR-AaUSP binds to other direct repeats separated by more than 10 nucleotides (DR-11 and DR-13) but with considerably lower affinity than to DR-12. Presently, it is not known whether insect EcR-USP heterodimers are capable of utilizing widely spaced half-sites (e.g., >100 bp) as response elements.

We observed that the ng EcRE, previously described as a DR-12, is a composite of three half-sites, suggesting the possibility that an internal 5'–proximal DR-2 and/or 3'–distal DR-4 might contribute to the functionality of this element. Mutating the 5' half-site in this element dramatically reduced its binding affinity, revealing its critical role in EcR-USP binding. In contrast, mutating the 3' half-site decreased binding affinity only slightly, whereas mutating the middle half-site did not have any obvious effect on the binding of the element. Thus, both competition and direct binding analyses of mutated ng EcRE suggest that in addition to functioning as a true DR-12, the ng element may also have a functional imperfect DR-2 located at its 5' end (Fig. 7).

Transactivation assays in CV-1 cells confirmed the finding of the DNA binding assays, demonstrating that the AaEcR-AaUSP heterodimer is indeed the functional edecysone receptor. Co-transfection of AaEcR and AaUSP expression vectors into CV-1 cells conferred 40-fold induction of the reporter plasmid ΔMTV-5xIRhop-1-CAT and 9-fold induction of ΔMTV-3xDR4-CAT in response to 1 μM MurA. We observed that the number of EcREs in a reporter construct is not directly proportional to the magnitude of reporter transactivation. A reporter plasmid containing a single copy of IRhop-1 was induced only 2.5-fold compared with a 40-fold induction of the reporter containing five copies of the same EcRE. Therefore, in order to compare transactivation activities of IR and DR elements, we utilized the reporter plasmids containing only one copy of either IRhop-1, DR-4, or IRper-1. Importantly, all three response elements were able to mediate edecysone responsiveness of the reporter in CV-1 cells. The transactivation efficiencies of tested response element followed the order IRper-1 > DR-4 > IRhop-1. Thus, despite the fact that the differences were not as dramatic as those for binding affinities measured for the same elements, the two sets of data are in agreement with one another (Table I). By using transfection in Drosophila Schneider-3 cells and endogenous receptor pools, Martinez et al. (53) also showed that for DmEcR-DmUSP, IRper-1 was transactivated about twice as well as IRhop-1 when placed before the thymidine kinase promoter. By using four copies of EcREs ahead of the hsp70 promoter, Vogtli et al. (54) reported that IRper-1 activated the reporter gene twice stronger than IRhop-1 and DR-4 in the presence of endogenous DmEcR-DmUSP in S2 cells. These results agree with our observations for CV-1 cells.

DR-4, the optimal DR for binding to AaEcR, was induced only 2.5-fold compared with a 40-fold induction of the reporter containing five copies of the same EcRE. Therefore, in order to compare transactivation activities of IR and DR elements, we utilized the reporter plasmids containing only one copy of either IRhop-1, DR-4, or IRper-1. Importantly, all three response elements were able to mediate edecysone responsiveness of the reporter in CV-1 cells. The transactivation efficiencies of tested response element followed the order IRper-1 > DR-4 > IRhop-1. Thus, despite the fact that the differences were not as dramatic as those for binding affinities measured for the same elements, the two sets of data are in agreement with one another (Table I). By using transfection in Drosophila Schneider-3 cells and endogenous receptor pools, Martinez et al. (53) also showed that for DmEcR-DmUSP, IRper-1 was transactivated about twice as well as IRhop-1 when placed before the thymidine kinase promoter. By using four copies of EcREs ahead of the hsp70 promoter, Vogtli et al. (54) reported that IRper-1 activated the reporter gene twice stronger than IRhop-1 and DR-4 in the presence of endogenous DmEcR-DmUSP in S2 cells. These results agree with our observations for CV-1 cells.

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