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(54) Title: A GENE SWITCH COMPRISING AN ECDYSONE RECEPTOR

(57) Abstract
The invention relates to an insect steroid receptor protein which is capable of acting as a gene switch which is responsive to a chemical inducer enabling external control of the gene.
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A gene switch comprising an ecdysone receptor

The present invention relates to the identification and characterisation of insect steroid receptors from the Lepidoptera species *Heliothis virescens*, and the nucleic acid encoding therefor. The present invention also relates to the use of such receptors, and such nucleic acid, particularly, but not exclusively, in screening methods, and gene switches. By gene switch we mean a gene sequence which is responsive to an applied exogenous chemical inducer enabling external control of expression of the gene controlled by said gene sequence.

Lipophilic hormones such as steroids induce changes in gene expression to elicit profound effects on growth, cellular differentiation, and homeostasis. These hormones recognise intracellular receptors that share a common modular structure consisting of three main functional domains: a variable amino terminal region that contains a transactivation domain, a DNA binding domain, and a ligand binding domain on the carboxyl side of the molecule. The DNA binding domain contains nine invariant cysteines, eight of which are involved in zinc coordination to form a two-finger structure. In the nucleus the hormone-receptor complex binds to specific enhancer-like sequences called hormone response elements (HREs) to modulate transcription of target genes.

The field of insect steroid research has undergone a revolution in the last three years as a result of the cloning and preliminary characterisation of the first steroid receptor member genes. These developments suggest the time is ripe to try to use this knowledge to improve our tools in the constant fight against insect pests. Most of the research carried out on the molecular biology of the steroid receptor superfamily has been on *Drosophila melanogaster* (Diptera), see for example International Patent Publication No WO91/13167, with some in *Manduca* and *Galleria* (Lepidoptera).

It has been three decades since 20-hydroxyecdysone was first isolated and shown to be involved in the regulation of development of insects. Since then work has been carried out to try to understand the pathway by which this small hydrophobic molecule regulates a number of activities. By the early 1970s, through the studies of Clever and Ashburner, it was clear that at least in the salivary glands of third instar *Drosophila* larvae, the application of ecdysone lead to the reproducible activation of over a hundred genes. The ecdysone receptor in this pathway is involved in the regulation of two classes of genes: a small class (early genes) which are induced by the ecdysone receptor and a large class (late genes) which are repressed by the ecdysone receptor. The early class of genes are thought to have two functions reciprocal to those of the ecdysone receptor; the repression of the early transcripts and the induction of late gene transcription. Members of the early genes so far isolated and characterised belong to the class of molecules with characteristics similar to known
transcription factors. They are thus predicted to behave as expected by the model of ecdysone action (Ashburner, 1991). More recently, the early genes E74 and E75 have been shown to bind both types of ecdysone inducible genes (Thummel et al., 1990; Segrevas and Hogness, 1991), thus supporting their proposed dual activities. It should be noted however, that the activation of a hierarchy of genes is not limited to third instar larvae salivary glands, but that the response to the ecdysone peak at the end of larval life is observed in many other tissues, such as the imaginal disks (i.e. those tissues which metamorphose to adult structures) and other larval tissues which histolyse at the end of larval life (e.g. larval fat body). The model for ecdysone action as deduced by studying the third instar chromosome puffing may not apply to the activation of ecdysone regulated genes in adults. In other words, the requirement for other factors in addition to the active ecdysone receptor must be satisfied for correct developmental expression (e.g. the Drosophila yolk protein gene expression in adults is under control of doublesex, the last gene in the sex determination gene hierarchy).

The ecdysone receptor and the early gene E75 belong to the steroid receptor superfamily. Other Drosophila genes, including ultraspiracle, tailless, sevenup and FTZ-FI, also belong to this family. However, of all these genes only the ecdysone receptor is known to have a ligand, and thus the others are known as orphan receptors. Interestingly, despite the ultraspiracle protein ligand binding region sharing 49% identity with the vertebrate retinoic X receptor (RXR) ligand binding region (Oro et al., 1990), they do not share the same ligand (i.e. the RXR ligand is 9-cis retinoic acid) (Heymann et al., 1992 and Mangelsdorf et al., 1992). All the Drosophila genes mentioned are involved in development, ultraspiracle for example, is required for embryonic and larval abdominal development. The protein products of these genes all fit the main features of the steroid receptor superfamily (Evans, 1988; Green and Chambon, 1988, Beato, 1989) i.e. they have a variable N terminus region involved in ligand independent transactivation (Domains A and B), a highly conserved 66-68 amino acid region which is responsible for the binding of DNA at specific sites (Domain C), a hinge region thought to contain a nuclear translocation signal (Domain D), and a well conserved region containing the ligand binding region, transactivation sequences and the dimerisation phase (Domain E). The last region, domain F, is also very variable and its function is unknown.

Steroid receptor action has been elucidated in considerable detail in vertebrate systems at both the cellular and molecular levels. In the absence of ligand, the receptor molecule resides in the cytoplasm where it is bound by Hsp90, Hsp70, and p59 to form the inactive complex (Evans, 1988). Upon binding of the ligand molecule by the receptor a conformational change takes place which releases the Hsp90, Hsp70 and p59 molecules, while exposing the nuclear translocation signals in the receptor. The ligand dependent conformational change is seen in the ligand binding domain of both progesterone and retinoic acid receptors (Allan et
al., 1992a). This conformational change has been further characterised in the progesterone receptor and was found to be indispensable for gene transactivation (Allan et al., 1992b). Once inside the nucleus the receptor dimer binds to the receptor responsive element at a specific site on the DNA resulting in the activation or repression of a target gene. The receptor responsive elements usually consist of degenerate direct repeats, with a spacer between 1 and 5 nucleotides, which are bound by a receptor dimer through the DNA binding region (Domain C).

Whereas some steroid hormone receptors are active as homodimers others act as heterodimers. For example, in vertebrates, the retinoic acid receptor (RAR) forms heterodimers with the retinoic X receptor (RXR). RXR can also form heterodimers with the thyroid receptor, vitamin D receptor (Yu et al., 1991; Leid et al., 1992) and peroxisome activator receptor (Kliwer et al., 1992). Functionally the main difference between homodimers and heterodimers is increased specificity of binding to specific response elements. This indicates that different pathways can be linked, co-ordinated and modulated, and more importantly this observation begins to explain the molecular basis of the pleiotropic activity of retinoic acid in vertebrate development (Leid et al., 1992b). Similarly, the Drosophila ultraspireacle gene product was recently shown to be capable of forming heterodimers with retinoic acid, thyroid, vitamin D and peroxisome activator receptors and to stimulate the binding of these receptors to their target responsive elements (Yao et al., 1993).

More significantly, the ultraspireacle gene product has also been shown to form heterodimers with the ecdysone receptor, resulting in cooperative binding to the ecdysone response element and capable of rendering mammalian cells ecdysone responsive (Yao et al., 1992). The latter is of importance since transactivation of the ecdysone gene alone in mammalian cells fails to elicit an ecdysone response (Koelle et al., 1991), therefore suggesting that the ultraspireacle gene product is an integral component of a functional ecdysone receptor (Yao et al., 1992). It is possible that the ultraspireacle product competes with other steroid receptors or factors to form heterodimers with the ecdysone receptor. Moreover it remains to be investigated if ultraspireacle is expressed in all tissues of the Drosophila larvae. Despite ultraspireacle being necessary to produce a functional ecdysone receptor, the mechanism by which this activation takes place is as yet undetermined.

We have now isolated and characterised the ecdysone steroid receptor from Heliothis virescens (hereinafter HEcR). We have found that surprisingly unlike the Drosophila ecdysone steroid receptor (hereinafter DEcR), in reports to-date, HEcR can be induced by known non-steroidal inducers. It will be appreciated that this provides many advantages for the system.

Steroids are difficult and expensive to make. In addition, the use of a non-steroid as the inducer allows the system to be used in agrochemical and pharmaceutical applications, not
least because it avoids application of a steroid which is already present in insects and/or mammals. For example, it would not be feasible to use a gene switch in a mammalian cell which was induced by a naturally occurring steroidal inducer. It will also be appreciated that for environmental reasons it is advantageous to avoid the use of steroids as inducers.

According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 2, wherein Seq ID No 2 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 2, which encodes for the HEcR carboxy terminal region.

According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 3, wherein Seq ID No 3 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 3, which encodes for the HEcR carboxy terminal region.
According to one aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 4, wherein Seq ID No 4 gives the sequence for the HEcR.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR transactivation domain.

According to a further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR hinge domain.

According to a still further aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 4, which encodes for the HEcR carboxy terminal region.

As mentioned above, steroid receptors are eukaryotic transcriptional regulatory factors which, in response to the binding of the steroid hormone, are believed to bind to specific DNA elements and activate transcription. The steroid receptor can be divided into six regions, designated A to F, using alignment techniques based on shared homology with other members of the steroid hormone receptor superfamily. Krust et al identified two main regions in the receptor, C and E. Region C is hydrophilic and is unusual in its high content in cysteine, lysine and arginine. It corresponds to a DNA-binding domain, sometimes referred to as the "zinc finger". It is the DNA binding domain which binds to the upstream DNA of the responsive gene. Such upstream DNA is known as the hormone response element or HRE for short. Region E is hydrophobic and is identified as the hormone (or ligand) binding domain. Region E can be further subdivided into regions E1, E2 and E3.

The region D, which separates domains C and E is highly hydrophobic and is flexible. It is believed that communication between domains E and C involves direct contact between them through region D, which provides a hinge between the two domains. Region D is therefore referred to as the hinge domain.

The mechanism of the receptor appears to require it to interact with some element(s) of the transcription machinery over and above its interactions with the hormone and the hormone response element. N-terminal regions A and B perform such a function and are jointly known as the transactivation domain. The carboxy terminal region is designated F.

The domain boundaries of the HEcR can be defined as follows:
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</table>

The DNA binding domain is very well defined and is 66 amino acids long, thus providing good boundaries. The above intervals have been defined using the multiple alignment for the ecdysone receptors (Figure 5).

The present invention also includes DNA which shows homology to the sequences of the present invention. Typically homology is shown when 60% or more of the nucleotides are common, more typically 65%, preferably 70%, more preferably 75%, even more preferably 80% or 85%, especially preferred are 90%, 95%, 98% or 99% or more homology.

The present invention also includes DNA which hybridises to the DNA of the present invention and which codes for at least part of the Heliotothis ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region. Preferably such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SCC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SCC is three time as strong as SSC and so on.

The present invention further includes DNA which is degenerate as a result of the genetic code to the DNA of the present invention and which codes for a polypeptide which is at least part of the Heliotothis ecdysone receptor transactivation domain, DNA binding domain, hinge domain, ligand binding domain and/or carboxy terminal region.

The DNA of the present invention may be cDNA or DNA which is in an isolated form.

According to another aspect of the present invention there is provided a polypeptide comprising the Heliotothis ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by any of the DNA of the present invention.

According to another aspect of the present invention there is provided a polypeptide which has the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, wherein Seq ID No. 4 gives the amino acid sequence of the HEcR polypeptide.
According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR ligand binding domain.

According to another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR DNA binding domain.

According to yet another aspect of the present invention there is provided a polypeptide which has part of the amino acid sequence of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR transactivation domain.

According to a further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR hinge domain.

According to a still further aspect of the present invention there is provided a polypeptide which has the amino acid sequence of a part of Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the HEcR carboxy terminal region.

For the avoidance of doubt, spliced variants of the amino acid sequences of the present invention are included in the present invention.

Preferably, said derivative is a homologous variant which has conservative amino acid changes. By conservation amino acid changes we mean replacing an amino acid from one of the amino acid groups, namely hydrophobic, polar, acidic or basic, with an amino acid from within the same group. An examples of such a change is the replacement of valine by methionine and vice versa.

According to another aspect of the present invention there is provided a fusion polypeptide comprising at least one of the polypeptides of the present invention functionally linked to an appropriate non-Helicotheris ecdysonic receptor domain(s).

According to an especially preferred embodiment of the present invention the HEcR ligand binding domain of the present invention is fused to a DNA binding domain and a transactivation domain.

According to another embodiment of the present invention the DNA binding domain is fused to a ligand binding domain and a transactivation domain.

According to yet another embodiment of the present invention the transactivation domain is fused to a ligand binding domain and a DNA binding domain.

The present invention also provides recombinant DNA encoding for these fused polypeptides.

According to an especially preferred embodiment of the present invention there is provided recombinant nucleic acid comprising a DNA sequence encoding the HEcR ligand
binding domain functionally linked to DNA encoding the DNA binding domain and transactivation domain from a glucocorticoid receptor.

According to yet another aspect of the present invention there is provided recombinant nucleic acid comprising a DNA sequence comprising a reporter gene operably linked to a promoter sequence and a hormone response element which hormone response element is responsive to the DNA bonding domain encoded by the DNA of the present invention.

According to another aspect of the present invention there is provided a construct transformed with nucleic acid, recombinant DNA, a polypeptide or a fusion polypeptide of the present invention. Such constructs include plasmids and phages suitable for transforming a cell of interest. Such constructs will be well known to those skilled in the art.

According to another aspect of the present invention there is provided a cell transformed with nucleic acid, recombinant DNA, a polypeptide, or a fusion polypeptide of the present invention.

Preferably the cell is a plant, fungus or mammalian cell.

For the avoidance of doubt fungus includes yeast.

The present invention therefore provides a gene switch which is operably linked to a foreign gene or a series of foreign genes whereby expression of said foreign gene or said series of foreign genes may be controlled by application of an effective exogenous inducer.

Analogs of ecdysone, such as Muristerone A, are found in plants and disrupt the development of insects. It is therefore proposed that the receptor of the present invention can be used be in plants transformed therewith as an insect control mechanism. The production of the insect-damaging product being controlled by an exogenous inducer. The insect-damaging product can be ecdysone or another suitable protein.

The first non-steroidal ecdysteroid agonists, dibenzoyl hydrazines, typified by RH-5849 [1,2-dibenzoyl, 1-tert-butyl hydrazide], which is commercially available as an insecticide from Rohm and Haas, were described back in 1988. Another commercially available compound in this series is RH-5992 [tebufenozide, 3,5-dimethylbenzoic acid 1-1 (1,1-dimethylethyl)-2(4-ethylbenzoyl) hydrazide]. These compounds mimic 20-hydroxyecdysone (20E) in both Manduca sexta and Drosophila melanogaster. These compounds have the advantage that they have the potential to control insects using ecdysteroid agonists which are non-steroidal. Further Examples of such dibenzoyl hydrazines are given in US Patent No. 5,117,057 to Rohm and Haas, and Oikawa et al, Pestic Sci, 41, 139-148 (1994). However, it will be appreciated that any inducer of the gene switch of the present invention, whether steroidal or non-steroidal, and which is currently or becomes available, may be used.
The gene switch of the present invention, then, when linked to an exogenous or foreign gene and introduced into a plant by transformation, provides a means for the external regulation of expression of that foreign gene. The method employed for transformation of the plant cells is not especially germane to this invention and any method suitable for the target plant may be employed. Transgenic plants are obtained by regeneration from the transformed cells. Numerous transformation procedures are known from the literature such as agroinfection using Agrobacterium tumefaciens or its Ti plasmid, electroporation, microinjection or plants cells and protoplasts, microprojectile transformation, to mention but a few. Reference may be made to the literature for full details of the known methods.

Neither is the plant species into which the chemically inducible sequence is inserted particularly germane to the invention. Dicotyledonous and monocotyledonous plants can be transformed. This invention may be applied to any plant for which transformation techniques are, or become, available. The present invention can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, and cotton; cereals such as wheat, barley, rice, maize, and sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas and melons; and vegetables such as carrot, lettuce, cabbage and onion. The switch is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

In a particularly preferred embodiment of the present invention, the gene switch of the present invention is used to control expression of genes which confer resistance herbicide resistance and/or insect tolerance to plants.

Recent advances in plant biotechnology have resulted in the generation of transgenic plants resistant to herbicide application, and transgenic plants resistant to insects. Herbicide tolerance has been achieved using a range of different transgenic strategies. One well documented example in the herbicide field is the use the bacterial xenobiotic detoxifying gene phosphinothricin acetyl transferase (PAT) from Streptomyces hygroscopicus. Mutated genes of plant origin, for example the altered target site gene encoding acetolactate synthase (ALS) from Arabidopsis, have been successfully utilised to generate transgenic plants resistant to herbicide application. The PAT and ALS genes have been expressed under the control of strong constitutive promoter. In the field of insecticides, the most common example to-date is the use of the Bt gene.

We propose a system where genes conferring herbicide and/or insect tolerance would be expressed in an inducible manner dependent upon application of a specific activating chemical. This approach has a number of benefits for the farmer, including the following:

1. Inducible control of herbicide and/or insect tolerance would alleviate any risk of yield penalties associated with high levels of constitutive expression of herbicide and/or insect resistance genes. This may be a particular problem as early stages of growth
where high levels of transgene product may directly interfere with normal
development. Alternatively high levels of expression of herbicide and/or insect
resistance genes may cause a metabolic drain for plant resources.

2. The expression of herbicide resistance genes in an inducible manner allows the
herbicide in question to be used to control volunteers if the activating chemical is
omitted during treatment.

3. The use of an inducible promoter to drive herbicide and/or insect resistance genes will
reduce the risk of resistance becoming a major problem. If resistance genes were
passed onto weed species from related crops, control could still be achieved with the
herbicide in the absence of inducing chemical. This would particularly be relevant if
the tolerance gene confirmed resistance to a total vegetative control herbicide which
would be used (with no inducing chemical) prior to sowing the crop and potentially
after the crop has been harvested. For example, it can be envisaged that herbicide
resistance cereals, such as wheat, might outcross into the weed wild oats, thus
confering herbicide resistance to this already troublesome weed. A further example is
that the inducible expression of herbicide resistance in sugar beet will reduce the risk
of wild sugar beet becoming a problem. Similarly, in the field of insect control, insect
resistance may well become a problem if the tolerance gene is constitutively expressed.
The used of an inducible promoter will allow a greater range of insect resistance
control mechanisms to be employed.

This strategy of inducible expression of herbicide resistance can be achieved with a
pre-spray of chemical activator or in the case of slow acting herbicides, for example N-
phosphonomethyl-glycine (commonly known as glyphosate), the chemical inducer can be
added as a tank mix simultaneously with the herbicide. Similar strategies can be employed for
insect control.

This strategy can be adopted for any resistance conferring gene/corresponding
herbicide combination, which is, or becomes, available. For example, the gene switch of the
present invention can be used with:

1. Maize glutathione S-transferase (GST-27) gene (see our International Patent
Publication No WO90/08826), which confers resistance to chloroacetanilide
herbicides such as acetochlor, metolachlor and alachlor.

2. Phosphinotricin acetyl transferase (PAT), which confers resistance to the herbicide
commonly known as glufosinate.

3. Acetolactate synthase gene mutants from maize (see our International Patent
Publication No WO90/14000) and other genes, which confer resistance to sulphonyl
urea and imidazolinones.
4. Genes which confer resistance to glyphosate. Such genes include the glyphosate oxidoreductase gene (GOX) (see International Patent Publication No. WO92/00377); genes which encode for 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS), including Class I and Class II EPSPS, genes which encode for mutant EPSPS, and genes which encode for EPSPS fusion peptides such as that comprised of a chloroplast transit peptide and EPSPS (see for example EP 218 571, EP 293 358, WO91/04323, WO92/04449 and WO92/06201); and genes which are involved in the expression of CPLyase.

Similarly, the strategy of inducible expression of insect resistance can be adopted for any tolerance conferring gene which is, or becomes, available.

The gene switch of the present invention can also be used to controlled expression of foreign proteins in yeast and mammalian cells. Many heterologous proteins for many applications are produced by expression in genetically engineered bacteria, yeast cells and other eucaryotic cells such as mammalian cells.

As well as the obvious advantage in providing control over the expression of foreign genes in such cells, the switch of the present invention provides a further advantage in yeasts and mammalian cells where accumulation of large quantities of an heterologous protein can damage the cells, or where the heterologous protein is damaging such that expression for short periods of time is required in order to maintain the viability of the cells.

Such an inducible system also has applicability in gene therapy allowing the timing of expression of the therapeutic gene to be controlled. The present invention is therefore not only applicable to transformed mammalian cells but also to mammals per se.

A further advantage of the inducible system of the present invention in mammalian cells is that, because it is derived from an insect, there is less chance of it being effected by inducers which effect the natural mammalian steroid receptors.

In another aspect of the present invention the gene switch is used to switch on genes which produce potentially damaging or lethal proteins. Such a system can be employed in the treatment of cancer in which cells are transformed with genes which express proteins which are lethal to the cancer. The timing of the action of such proteins on the cancer cells can be controlled using the switch of the present invention.

The gene switch of the present invention can also be used to switch genes off as well as on. This is useful in disease models. In such a model the cell is allowed to grow before a specific gene(s) is switched off using the present invention. Such a model facilitates the study of the effect of a specific gene(s).

Again the method for producing such transgenic cells is not particularly germane to the present invention and any method suitable for the target cell may be used; such methods are known in the art, including cell specific transformation.
As previously mentioned, modulation of gene expression in the system appears in response to the binding of the HEcR to a specific control, or regulatory, DNA element. A schematic representation of the HEcR gene switch is shown in Figure 6. For ease of reference, the schematic representation only shows three main domains of the HEcR, namely the transactivation domain, DNA binding domain and the ligand binding domain. Binding of a ligand to the ligand binding domain enables the DNA binding domain to bind to the HRE resulting in expression (or indeed repression) of a target gene.

The gene switch of the present invention can therefore be seen as having two components. The first component comprising the HEcR and a second component comprising an appropriate HRE and the target gene. In practice, the switch may conveniently take the form of one or two sequences of DNA. At least part of the one sequence, or one sequence of the pair, encoding the HEcR protein. Alternatively, the nucleic acid encoding the HEcR can be replaced by the protein/ polypeptide itself.

Not only does the switch of the present invention have two components, but also one or more of the domains of the receptor can be varied producing a chimeric gene switch. The switch of the present invention is very flexible and different combinations can be used in order to vary the result/to optimise the system. The only requirement in such chimeric systems is that the DNA binding domain should bind to the hormone response element in order to produce the desired effect.

The glucocorticoid steroid receptor is well characterised and has been found to work well in plants. A further advantage of this receptor is that it functions as a homodimer. This means that there is no need to express a second protein such as the ultraspiracle in order to produce a functional receptor. The problem with the glucocorticoid steroid receptor is that ligands used to activate it are not compatible with agronomic practice.

In a preferred aspect of the present invention the receptor comprises glucocorticoid receptor DNA binding and transactivation domains with a *Heliothis* ligand binding domain according to the present invention. The response unit preferably comprising the glucocorticoid hormone response element and the desired effect gene. In the Examples, for convenience, this effect gene took the form of a reporter gene. However, in non-test or non-screen situations the gene will be the gene which produces the desired effect, for example produces the desired protein. This protein may be a natural or exogenous protein. It will be appreciated that this chimeric switch combines the best features of the glucocorticoid system, whilst overcoming the disadvantage of only being inducible by a steroid.

In another preferred embodiment, the *Heliothis* ligand binding domain is changed, and preferably replaced with a non-*Heliothis* ecdysone receptor ligand binding domain. For example, we have isolated suitable sequences from Spodoptera exigua.
Thus, according to another aspect of the present invention there is provided DNA having the sequence shown in Seq ID No. 6.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 6, which encodes for the *Spodoptera* ecdysone ligand binding domain.

According to another aspect of the present invention there is provided DNA having part of the sequence shown in Seq ID No. 6, which encodes for the *Spodoptera* ecdysone hinge domain.

The present invention also provides the polypeptides coded for by the above DNA sequences of Seq ID No. 6.

A further advantage with such chimeric systems is that they allow you to choose the promoter which is used to drive the effector gene according to the desired end result. For example, placing the foreign gene under the control of a cell specific promoter can be particularly advantageous in circumstances where you wish to control not only the timing of expression, but also which cells expression occurs in. Such a double control can be particularly important in the areas of gene therapy and the use of cytotoxic proteins.

Changing the promoter also enables gene expression to be up- or down-regulated as desired.

Any convenient promoter can be used in the present invention, and many are known in the art.

Any convenient transactivation domain may also be used. The transactivation domain VP16 is a strong activator from Genentech Inc., and is commonly used when expressing glucocorticoid receptor in plants. Other transactivation domains derived for example from plants or yeast may be employed.

In a preferred embodiment of the present invention, the DNA binding domain is the glucocorticoid DNA binding domain. This domain is commonly a human glucocorticoid receptor DNA binding domain. However, the domain can be obtained from any other convenient source, for example, rats.

According to another aspect of the present invention there is provided a method of selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to a polypeptide or fusion polypeptide of the present invention, and selecting said compounds exhibiting said binding.

According to another aspect of the present invention there is provided a compound selected using the method of the present invention.

According to another aspect of the present invention there is provided an agricultural or pharmaceutical composition comprising the compound of the present invention.
According to yet another aspect of the present invention there is provided the use of the compound of the present invention as a pesticide, pharmaceutical and/or inducer of the switch. It will be appreciated that such inducers may well be useful as insecticides in themselves.

According to a further aspect of the present invention there is provided a method of producing a protein or peptide or polypeptide comprising introducing into a cell of the present invention, a compound which binds to the ligand binding domain in said cell.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example with reference to the accompanying examples and figures, in which figures:

Figure 1 (Sequence ID No. 1) shows the DNA sequence amplified from first strand cDNA made from mRNA isolated from *Heliothis virescens* Fourth instar larvae. The underlined sequences refer to the position of the degenerate oligonucleotides. At the 5' end the sequence matches that of the oligonucleotide while at the 3' end 12 nucleotides of the original oligonucleotide are observed;

Figure 2 (Sequence ID No. 2) shows the DNA sequence contained within the clone pSK19R isolated from a random primed cDNA *Heliothis virescens* library; Sequence is flanked by EcoRI sites;

Figure 3 (Sequence ID No. 3) shows the DNA sequence contained within the clone pSK16.1 isolated from a random primed cDNA *Heliothis virescens* library;

Figure 4 (Sequence ID No. 4) DNA sequence of 5'RACE products (in bold) fused to sequence of clone pSK16.1. The ORF (open reading frame) giving rise to the *Heliothis virescens* ecdysone receptor protein sequence is shown under the corresponding DNA sequence;

Figure 5 (Sequence ID No. 5) shows the protein sequence alignment of the ecdysone receptors DmEcR (*Drosophila melanogaster*), CtEcR (*Chironomus tentans*), BmEcR (*Bombyx mori*), MsEcR (*Manduca sexta*), AaEcR (*Aedes aegypti*) and HvEcR (*Heliothis virescens*). "*" indicates conserved amino acid residue. "." indicates a conservative amino acid exchange;

Figure 6 shows a model of an embodiment of the glucocorticoid/*Heliothis* ecdysone chimeric receptor useable as a gene switch;

Figure 7 shows a plasmid map of the clone pCDNA319R. The three other mammalian expression vectors were constructed in the same way and look similar but for the size of the insert;

Figure 8 shows a plasmid map of the reporter construct used to analyse the activity of the *Heliothis virescens* ecdysone receptor;
Figure 9 is a graph which shows the effect of Muristerone A and RH5992 in reporter activity in HEK293 cells co-transfected with pcDNA3H3KHEcR alone (filled bars) or with αRXR (stripped bars);

Figure 10 shows a plasmid map of the Maize expression vector containing the Glucocorticoid receptor (HG1 or pMF6HG1PAT);

Figure 11 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/Drosophila ecdysone receptor pMF6GREcRS;

Figure 12 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid/Heliothis ecdysone receptor pMF6GRHecR;

Figure 13 shows a plasmid map of the plant reporter Plasmid containing the glucocorticoid response elements fused to the -60 S35CaMV promoter fused to GUS, p221.9GRE6;

Figure 14 shows a plasmid map of the plant reporter plasmid containing the glucocorticoid response elements fused to the -46 S35CaMV promoter fused to GUS, p221.10GRE6;

Figure 15 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6HG1PAT (GR) and p221.9GRE6 (reporter);

Figure 16 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 17 shows a graph showing the effect of Muristerone A and Dexamethasone in Maize AXB protoplasts transformed with pMF6GRHecR (effector) and p221.9GRE6 (reporter);

Figure 18 shows a graph showing the effect of RH5849 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 19 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GREcRS (effector) and p221.9GRE6 (reporter);

Figure 20 shows a graph showing the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHecR (effector) and p221.9GRE6 (reporter);

Figure 21 shows a graph which shows the dose response effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRHecR (effector) and p221.9GRE6 (reporter);

Figure 22 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/ Drosophila ecdysone receptor, pMF7GREcRS;

Figure 23 shows a plasmid map of the tobacco expression vector containing the chimeric glucocorticoid/ Heliothis ecdysone receptor, pMF7GRHecR;

Figure 24 shows a graph which shows the effect of RH5992 in Tobacco mesophyll protoplasts transformed with pMF6GRHecR (Effector) and p221.9GRE6 (reporter);
Figure 25 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid/Heliothis ecdysone receptor, pcDNA3GRHEcR;

Figure 26 shows a plasmid map of the reporter plasmid pSWGREG4;

Figure 27 shows a graph which shows a RH5992 dose response curve of CHO cells transfected with pcDNA3GRHEcR and pSWGREG4;

Figure 28 shows a graph which shows the effect of Muristerone A and RH5992 on HEK293 cells co-transfected with pcDNA3GRHEcR and pSWGREG4;

Figure 29 shows a plasmid map of the binary vector ES1;

Figure 30 shows a plasmid map of the binary vector ES2;

Figure 31 shows a plasmid map of the binary vector ES3;

Figure 32 shows a plasmid map of the binary vector ES4;

Figure 33 shows a plasmid map of the effector construct TEV-B112 made to express the HEcR ligand binding domain in yeast;

Figure 34 shows a plasmid map of the effector construct TEV8 made to express the HEcR ligand binding domain in yeast;

Figure 35 shows a plasmid map of the effector construct TEVVP16-3 made to express the HEcR ligand binding domain in yeast;

Figure 36 shows a plasmid map of the mammalian expression vector containing the chimeric glucocorticoid VP16/Heliothis ecdysone receptor, pcDNA3GRVP16HEcR;

Figure 37 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid VP16/Heliothis ecdysone receptor, pMF6GRVP16HEcR;

Figure 38 shows a plasmid map of the maize expression vector containing the chimeric glucocorticoid VP16/Heliothis ecdysone receptor, pMF7GRVP16HEcR;

Figure 39 shows a graph which shows the effect of RH5992 in Maize AXB protoplasts transformed with pMF6GRVP16HEcR (effector) and p221.9GRE6 (reporter);

Figure 40 (Sequence ID No. 6) shows the DNA sequence of the hinge and ligand binding domains of the Spodoptera exigua ecdysone receptor;

Figure 41 (Sequence ID No. 7) shows the protein sequence alignment of the Heliothis 19R and Spodoptera SEC R Taq clone hinge and ligand binding domains. "*" indicates conserved amino acid residue. "." indicates a conservative amino acid exchange;

Figure 42 shows a graph which shows the effect of RH5992 on Tobacco mesophyll protoplasts transformed with pMF7GRHEcR (effector) and either p221.9GRE6 (Horizontal strips) or p221.10GRE6 (vertical strips).
Example I - Cloning of the *Heliothis* Ecdysone Receptor

A. Probe generation

The rational behind the generation of the probe to isolate *Heliothis* homologues to the steroid/thyroid receptor superfamily members was based on comparing the sequences of developmentally regulated steroid/thyroid receptor superfamily members. The sequences available showed a highly conserved motif within the DNA binding domain of the RAR and THR (thyroid) receptors. The motifs were used to design degenerate oligonucleotides for PCR amplification of sequences derived from cDNA template produced from tissue expected to express developmentally regulated steroid/thyroid receptor superfamily members (ie. larval tissues).

The sense oligonucleotide is based on the peptide sequence CEGCKGFF which at the DNA level yields an oligonucleotide with degeneracy of 32 as shown below:

\[
\begin{align*}
\text{ZnFA} & \quad 5' \text{TGC GAG GGI TGC AAG GAI TTC TT 3'} \\
& \quad \text{T A T A T}
\end{align*}
\]

The antisense oligonucleotide is based on the reverse complement nucleotide sequence derived from the peptide:

\[
\begin{align*}
\text{CQECLRKK} \\
& \quad \text{S R}
\end{align*}
\]

for which four sets of degenerate oligos were made. Namely:

\[
\begin{align*}
\text{ZnFA} & \quad 5' \text{TTC TTI AGI CGGCAC TCT TGG CA 3'} \\
& \quad \text{T A T C A}
\end{align*}
\]

\[
\begin{align*}
\text{ZnFB} & \quad 5' \text{TTC TTI AAI CGGCAC TCT TGG CA 3'} \\
& \quad \text{T A T C A}
\end{align*}
\]

\[
\begin{align*}
\text{ZnFC} & \quad 5' \text{TTC TTI AGI CTGCAC TCT TGG CA 3'} \\
& \quad \text{T A T C A}
\end{align*}
\]

\[
\begin{align*}
\text{ZnFD} & \quad 5' \text{TTC TTI AAI CTGCAC TCT TGG CA 3'} \\
& \quad \text{T A T C A}
\end{align*}
\]

The PCR amplification was carried out using a randomly primed cDNA library made from mRNA isolated from 4th and 5th instar *Heliothis virescens* larvae. The amplification
was performed using 10^8 pfu (plaque forming units) in 50mM KCl, 20mM Tris HCl pH 8.4, 15mM MgCl2, 200mM dNTPs (an equimolar mixture of dCTP, dATP, dGTP and dTTP), 100ng of ZnFA5' and ZnF3' mixture. The conditions used in the reaction followed the hot start protocol whereby the reaction mixture was heated to 94°C for 5 minutes after which 1 U of Taq polymerase was added and the reaction allowed to continue for 35 cycles of 93°C for 50 seconds, 40°C for 1 minute and 73°C for 1 minute 30 seconds. The PCR products were fractionated on a 2%(w/v) agarose gel and the fragment migrating between 100 and 200bp markers was isolated and subcloned into the vector pCRII (Invitrogen). The sequence of the insert was determined using Sequenase (USB).

The resulting sequence was translated and a database search carried out. The search recovered sequences matching to the DNA binding domain of the *Drosophila* ecdysone receptor, retinoic acid receptor and the thyroid receptor. Thus, the sequence of the insert in this plasmid, designated pCRIIZnf, is a *Heliothis* ecdysone cognate sequence (Figure 1) and was used to screen a cDNA library in other to isolate the complete open reading frame.

B. Library screening

The randomly primed cDNA 4th/5th Instar *Heliothis virescens* library was plated and replicate filter made from the plates. The number of plaques plated was 500,000. The insert fragment of pCRIIZnf was reamplified and 50ng were end labelled using T4 Polynucleotide Kinase (as described in Sambrook et al 1990).

The filter were prehybridised using 0.25%(w/v) Marvel, 5 X SSPE and 0.1%(w/v) SDS at 42°C for 4 hours. The solution in the filters was ten replaced with fresh solution and the denatured probe added. The hybridisation was carried out overnight at 42°C after which the filter were washed in 6 X SSC + 0.1%(w/v) SDS at 42°C followed by another wash at 55°C. The filter were exposed to X-ray film (Kodak) for 48 hours before processing.

The developed film indicated the presence of one strong positive signal which was plaque purified and further characterised. The lambda ZAP II phage was in vivo excised (see Stratagene Manual) and the sequence determined of the resulting plasmid DNA. The clone known as pSK19R (or 19R) contained a 1.933kb cDNA fragment with an open reading frame of 467 amino acids (Figure 2). pSK19R was deposited with the NCIMB on 20 June 1995 and has been accorded the deposit No NCIMB 40743.

Further analysis of pSK19R revealed that a 340 bp EcoRI fragment mapping at the 5' end of pSK19R has strong and significant similarities to a *Drosophila* cDNA encoding glyceraldehyde-3-phosphate dehydrogenase. In order to isolate the correct 5'end sequence belonging to *Heliothis*, the random primed library was re-screened using a probe containing the 5'end of the pSK19R belonging to *Heliothis* ecdysone receptor. The probe was made by PCR using the sense oligonucleotide HecRH3C (5' aattaagttccacatgcggtaaactgcgcaccgaca
3') and antisense oligonucleotide HecrNdeI (5' cttcaacgcacactcttgac 3'). The PCR was carried out as described by Hirst et al., 1992 where the amount of radioisotope used in the labelling was 50uCi of a 32P-dCTP and the PCR was cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 19 cycles. The resulting 353bp radio labelled DNA fragment was denatured and added to prehybridised filters as described for the isolation of pSK19R. The library filters were made from 15 plates each containing 50000 pfus. The library filters were hybridised at 65°C and washed in 3XSSPE + 0.1%SDS at 65°C twice for 30 minutes each. The filters were further washed with 1XSSPE + 0.1%SDS for 30 minutes and exposed to X-ray film (Kodak) overnight. The film was developed and 16 putative positive plaques were picked. The plaques were re-plated and hybridised under the exact same conditions as the primary screen resulting in only one strong positive. The strong positive was consistently recognised by the probe and was plaque purified and in vivo excised. The resulting plasmid pSK16.1 was sequenced (Seq 1D3) which revealed that the 5' end of the clone extended by 205 bp and at the 3' end by 653 bp and resulting in a DNA insert of 2.5 kb. Conceptual translation of the 205 bp yielded 73 amino acids with high similarity to the Drosophila, Aedes aegiplti, Manduca and Bombyx sequences of the ecdysone receptor B1 isoform. However, the whole of the 5' end sequence is not complete since a Methionine start site was not found with a stop codon in frame 5' of the methionine. In order to isolate the remainder of the 5' end coding sequences a 5'RACE protocol (Rapid Amplification of cDNA Ends) was carried out using the BRL-GIBCO 5'RACE Kit. Two types of cDNA were synthesised where the first one used a specific oligonucleotide:

16PCR2A 5' cagctccagggccgctacgc3'

and the second type used random hexamers (oligonucleotide containing 6 random nucleotides). Each cDNA was PCR amplified using the oligonucleotides anchor primer:

BRL-GIBCO 5' caucauaauagccacggtcgactagtagcggiggiggig 3'

and 16PCR2A and cycled for 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C for 35 cycles. The reaction conditions were 20mM Tris-HCl (pH8.4), 50mM KCl, 1.5mM MgCl2, 400mM of each anchor and 16PCR2A primers, 200mM dNTPs (dATP,dCTP,dGTP and dTTP) and 0.02 U/ml Taq DNA polymerase. Dilutions of 1:50 of the first PCR reactions were made and 1ml was use in a second PCR with oligonucleotides UAP:

(Universal Amplification Primer 5' caucauaauagccacggtcgactagtc 3')

and 16RACE2:

(5' acgtcactccagagctctccattc 3').

The conditions and cycling were the same as those followed for the first PCR.

Samples of each PCR were run and a Southern blot carried out which was probed with a 5' specific primer:

(16PCR1 5' cgctggtagataaaacggagacattc 3').
This primer is specific for the 5' most sequence of pSK16.1 and was hybridised at 55°C using the standard hybridisation buffer. The filter was washed at 55°C 3 times in 3XSSPE + 0.1%SDS and exposed to X-ray film for up to 6 hours. The developed film revealed bands recognised by the oligonucleotide migrating at 100bp and 500bp (relative to the markers). A sample of the PCR reaction (4 in total) was cloned into the pCRII vector in the TA cloning kit (Invitrogen). Analysis of 15 clones from 4 independent PCRs yielded sequence upstream of pSK16.1 (Figure 4).

Translation of the ORF results in a 575 amino acid protein with high similarity in the DNA and ligand binding domains when compared to the ecdysone receptor sequences of *Drosophila*, *Aedes aegypti*, *Chironomus tentans*, *Manduca sexta* and *Bombyx mori* (Figure 5). Interestingly, the N-terminal end of the *Heliothis* sequence has an in frame methionine start which is 20 amino acids longer that that reported for *Drosophila*, *Aedes aegypti* and *Manduca sexta*. However, the extended N-terminal end in the *Heliothis* EcR does not have similarity to that of *Bombyx mori*. Finally, the C-terminal end of the different B1 isoform ecdysone receptor sequences diverge and do not have significant similarity.

C. Northern Blot Analysis

The sequence identified by screening the library is expected to be expressed in tissues undergoing developmental changes, thus mRNA from different developmental stages of H. virescens were isolated and a northern blot produced. The mRNAs were isolated from eggs, 1st, 2nd, 3rd, 4th and 5th instar larvae, pupae and adults. The northern blot was hybridised with a NdeI/XhoI DNA fragment from pSK19R encompassing the 3'end of the DNA binding domain through to the end of the ligand binding domain. The hybridisation was carried out in 1%(w/v)Marvel, 5X SSPE, 0.1%(w/v) SDS at 65°C for 18 to 24 hours. The filters were washed in 3X SSPE + 0.1%(w/v) SDS and 1X SSPE + 0.1%(w/v) SDS at 65°C. The filter was blotted dry and exposed for one to seven days. The gene recognises two transcripts (6.0 and 6.5 kb) which appear to be expressed in all stages examined, however, the levels of expression differ in different stages. It should be noted that the same two transcripts are recognised by probes specific to the DNA binding domain and the ligand binding domain, indicating that the two transcripts arise from the same gene either by alternative splicing or alternative use of polyadenylation sites.

In summary, adult and 5th instar larvae have lower levels of expression while all other tissues have substantial levels of expression.
Example II Expression of *Heliothis* ecdysone receptor in Mammalian cells

To demonstrate that the cDNA encodes a functional ecdysone receptor, effector constructs were generated containing the HEcR under the control of the CMV (cytomegalovirus) promoter, and the DNA expressed in mammalian cells.

Effector constructs

A first mammalian expression plasmid was constructed by placing a HindIII/NotI pSK19R fragment into the pcDNA3 HindIII/NotI vector resulting in pcDNA319R (Figure 7).

A second effector plasmid was constructed wherein the non-coding region of the cDNA 19R was deleted and a consensus Kozak sequence introduced. The mutagenesis was carried out by PCR amplifying a DNA fragment with the oligo HecRH3C:

5' attaaagctttccacctgacgcttacatagtcacgagc 3'

containing a unique HindIII restriction enzyme recognition site followed by the mammalian Kozak consensus sequence, and HecRNdeI:

5' cttcaacgcgactctgctg 3'.

The resulting 353bp PCR fragment was restriction enzyme digested with HindIII and NdeI, gel purified and ligated with 19R NdeI/NotI fragment into a pcDNA3 HindIII/NotI vector resulting in pcDNA3HecR.

A third effector construct was made with the 5' end sequences of pSK16.1 by PCR. The PCR approach involved PCR amplifying the 5' end sequences using a 5' oligonucleotide containing a HindIII restriction cloning site, the Kozak consensus sequence followed by nucleotide sequence encoding for a Methionine start and two Arginines to be added to the 5' end of the amplified fragment:

(16H3K 5' attaaagctttccacctgacgcttacatagtcacgagc 3'),
the 3' oligonucleotide used was HecRNdeI. The resulting fragment was restriction enzyme digested, gel purified and subcloned with an NdeI/NotI 19R fragment into pcDNA3 NdeI/NotI vector. The plasmid was named pcDNA3H3KHEcR.

A fourth effector construct was produced which contains the extended N-terminal end sequence obtained from the 5'RACE experiment. Thus, a PCR approach was followed to introduce the new 5' end sequences in addition to a consensus Kozak sequence and a HindIII unique cloning sequence. The sense oligonucleotide used was RACEH3K:

5' attaaagtgccgccattgcctgccgcttcgttgctggat 3',

while the antisense primer was the same as that used before (HecRNdeI). The cloning strategy was the same as used for the pcDNA3H3KHEcR to give rise to pcDNA3RACEH3KHEcR.

The PCR mutagenesis reactions were carried out in the same manner for all constructs. The PCR conditions used were 1 minute at 94°C, 1 minute at 60°C and 1 minute
at 72°C for 15 cycles. The reactions were 50mM Tris-HCl (pH8.4), 25mM KCl, 200mM dNTPs (dATP, dCTP, dGTP and dTTP), 200mM of each oligonucleotide and 2.5U/Reaction of Taq DNA polymerase. For each construct at least 5 independant PCR reactions were carried out and several clones were sequenced to insure that at least one is mutation free.

**Reporter construct**

The reporter plasmid to be co-transfected with the expression vector contained 4 copies of the Hsp27 ecdysone response element (Riddihough and Pelham, 1987) fused to B-globin promoter and the B-Galactosidase gene. The tandem repeats of the ecdysone response element were synthesised as two complementary oligonucleotides which when annealed produced a double stranded DNA molecule flanked by an SpeI site at the 5' end and a ClaI site at the 3' end:

Recr3A

5’ctagtagacaaggggttcaatgcacttgocaaataagcttagacaaggggttcaatgcacttgocaaataaacatgagacagacaaggggttcaattgcaaat 3’

Recr3B

5’cgattaggcaagtagtggcaaccccgtgtctctgcacagatggacaagtagtggcacttgggaaccccgttgtctgaattctagttgcagcattgg aacccctgtctagtattgggacaagtagtggcaaccccgtgtctctgcacagatggacaagtagtggcatttg 3’

The resulting 135bp DNA fragment was ligated to the vector pSWBGAL SpeI/ClaI resulting in pSWREcR4 (Figure 8). The co-transfection of the two plasmid should result in B-galactosidase activity in the presence of ligand. The experiment relies upon the presence of RXR (a homologue of ultraspiracle) in mammalian cells for the formation of an active ecdysone receptor.

**Mammalian transfection methods**

Transfections of mammalian cell lines (CHO-K1 Chinese hamster ovary)-ATCC number CCL61 or cos-1 (Monkey cell line) were performed using either calcium phosphate precipitation (Gorman, Chapter 6 of "DNA cloning: a practical approach. Vol 2 D.M. Glover ed./1985) IRL Press, Oxford) or using LipofectAMINE (Gibco BRL Cat. No. 18324-012, following manufacturers instructions). Human Epithelial Kidney 293 cells were transfected using analogous methods.

**Results - Native HEcR drives transient reporter gene expression in mammalian cells**

Co-transfection of pcDNA3H3KHEcR (Effector) and reporter constructs into Human Epithelial Kidney 293 cells (HEK293) in the presence of either Muristerone A or RH5992 resulted in a 2-3 fold induction of reporter activity compared to the no chemical controls (Figure 9). The HEK293 cells were used since they are known to have constitutive levels of αRXR which have been demonstrated to be necessary for *Drosophila* EcR activation by Muristerone A (Yao, *et al.*, 1993). Moreover, to further investigate the need for RXR
interactions, a αRXR was co-transfected into HEK293 cells (along with the effector and reporter) resulting in a 9 fold induction of reporter activity compared to the untreated cells (Figure 9). The co-transfection of αRXR with reporter and effector increased by four fold the reporter activity compared to cells transfected with effector and reporter alone. Induction was observed both in the presence of either Muristerone A or RH5992. These data clearly demonstrate that the cDNA HEcR encodes a functional ecdysone receptor. Moreover, the ability of HEcR to complex with αRXR and bind Muristerone A or RH5992 provide evidence for the usage of the entire HEcR as a component of a mammalian gene switch. In particular, it offers the advantage of reducing uninduced expression of target gene since ecdysone receptor and response elements are not present in mammalian cells.

Example III - Chimeric constructs and ligand validation in Maize Protoplasts

In order to apply the ecdysone receptor as an inducible system it was deemed necessary to simplify the requirements of the system by avoiding the need of a heterodimer formation to obtain an active complex. The glucocorticoid receptor is known to form homodimers and chimeric constructs of the glucocorticoid receptor transactivating and DNA binding domains fused to the ecdysone receptor hinge and ligand binding domains have been shown to be active as homodimers in mammalian cells in the presence of Muristerone A (an ecdysone agonist)(Christopherson et al., 1992). However, the chimeric receptor is not responsive to 20-hydroxyecdysone (Christopherson et al., 1992).

The analysis of the activation of the glucocorticoid/Heliotris ecdysone chimeric receptor entailed the production of two other control effector constructs. The first one of the constructs contained the intact glucocorticoid receptor while the second one contained a glucocorticoid/Drosophila ecdysone chimeric receptor.

**Effector constructs**

(i) Glucocorticoid receptor Maize expression construct

The glucocorticoid receptor DNA for the Maize transient expression construct was produced via the polymerase chain reaction (PCR) of Human Fibrosarcoma cDNA (HT1080 cell line, ATCC#CCI121) library (Clontech)(see Hollenberg et al., 1985). The PCR approach taken was to amplify the 2.7kb fragment encoding the glucocorticoid receptor in two segments. The first segment entails the N-terminal end up to and including the DNA binding domain while the second segment begins with the hinge region (amino acid 500) thought to the end of the reading frame. Thus, the PCR primer for the N-terminal end segment was designed to contain an EcoRI site and the Kozak consensus sequence for translation initiation:

```
GREcoRI 5'attgaatcaccatgacctcaagaatctaactc 3'.
```
The 3'end primer contains a XhoI site in frame with the reading frame at amino acid 500 of the published sequence:

GRXhoI 5' gagactctctgtagtgggtccgatggctcctttttttttttttttttttt 3'.

The second fragment of the glucocorticoid receptor was produced with a 5' end oligonucleotide containing an XhoI site in frame with the open reading frame at the beginning of the hinge region (amino acid 500):

GRHinge 5' attctcgagattcagccaggccactacagg 3'
while the 3' end oligonucleotide contained an EcoRI site 400 bp after the stop codon:

GRStop 5' attgaattcaatgtctagctactatacagg 3'.

The glucocorticoid receptor PCR was carried out using Vent polymerase (Biolabs) under hot start conditions followed by 15 cycles of denaturing (94°C for 1 minute), annealing (66°C for 1 minute) and DNA synthesis (72°C for 3 minute). The template was produced by making first strand cDNA as described in the TA cloning kit (Invitrogen) after which the PCR was carried out in 10mM KCl, 10mM (NH₄)₂SO₄, 20mM TRIS-HCl pH 8.8, 2 mM MgSO₄, 0.1% (v/v) Triton X-100, 200 mM dNTPs, 100ng of each Primer and 2 U of Vent Polymerase. The PCR products was restriction enzyme digested with EcoRI and XhoI and subcloned into pBluescript SK (pSK) EcoRI. The resulting plasmid pSKHGI was sequenced and found to lack any mutations from the published sequences (apart from those introduced in the PCR primers) (Hollenberg et al., 1985).

The 2.7kb EcoRI fragment was subcloned into the vector pMF6PAT EcoRI resulting in pMF6HGIPAT (Figure 10).

(ii) Maize expression construct containing a Glucocorticoid/ Drosophila ecdysone chimeric receptor.

The glucocorticoid receptor portion of the chimeric receptor was isolated from pSKHGI by producing a 1.5kb BamHI/XhoI restriction fragment containing the N-terminal end up to and including the DNA binding domain.

The Drosophila ecdysone receptor portion was isolated through PCR of first stand cDNA prepared from Drosophila adult mRNA. The PCR was carried out using a 5' oligonucleotide containing a SalI site (ie. Drosophila ecdysone receptor contains a XhoI site at the end of the ligand binding domain) which starts at the beginning of the hinge region:

amino acid 330, EcR8 attctcgaaacggccggaatgctgctccggag 3'.

The 3' end oligonucleotide contains an BamHI site adjacent to the stop codon:

EcRstop 5' tcgggtttaagatcctagccgtgccaagctgtccgacttaac 3'.

The PCR was carried out under the conditions described for the amplification of the Glucocorticoid receptor and yielded a 1.6 kb fragment. The fragment was introduced into
pSK Sall/BamHI and the sequence determined and compared to the published one (Koelle et al., 1991).

The maize transient expression plasmid was produced by introducing into pMF6 BamHI vector the 1.5kb BamHI/XhoI glucocorticoid receptor fragment and the 1.6kb Sall/BamHI Drosophila receptor portion to yield the chimeric plasmid pMF6GREcRS (Figure 9).

(iii) Construction of the Glucocorticoid/Heliothis ecdysone chimeric receptor: Maize transient expression plasmid.

The Glucocorticoid receptor portion of the chimera was produced as describe in Example II(ii). The production of the Heliothis ecdysone receptor portion involves the introduction of a Sall recognition site at the DNA binding/hinge domain junction (amino acid 229). The addition of the Sall site:

HehrsS 5'atgtcccagcagccgagtctgtgtgctgccgag 3'

was achieved via PCR mutagenesis making use of an unique AccI site 107bp downstream of the junction point (or 1007 bp relative to Seq 1D No 4):

Hehracc 5' tcacattgcatgagcaggtcagcgtccgag 3'.

The PCR was carried out using Taq polymerase (2.5 U) in a reaction buffer containing 100ng of template DNA (pSK19R), 100ng of Hehrs and Hehacc, 20 mM TRIS-HCl pH 8.4, 50mM KCl, 10mM MgCl₂, 200mM dNTPs. The reaction was carried out with an initial denaturation of 3 minutes followed by 15 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 60°C) and DNA synthesis (1 minute at 72°C). The DNA was restriction enzyme digested and subcloned into pSK Sall/SacI with the 1.2kb AccI/SacI 3' end HecR fragment to yield pSK HeCRDEF (or containing the hinge and ligand binding domains of the Heliothis ecdysone receptor).

The construction of the maize transient expression plasmid containing the Glucocorticoid/Heliothis ecdysone chimeric receptor involved the ligation of pMF6 EcoRI/SacI with the 1.5kb EcoRI/XhoI fragment of Glucocorticoid receptor N-terminal end and the 1.2 kb Sall/SacI fragment of pSk HECRDEF to yield pMF6GRHEcR (Figure 10).

Reporters plasmids

Two reporter plasmids were made by inserting the into p221.9 or p221.10 BamHI/HindIII vectors two pairs or oligonucleotides containing six copies of the glucocorticoid response element (GRE). The two sets of oligonucleotides were designed with restriction enzyme recognition sites so as to ensure insertion of the two pairs in the right orientation. The first oligonucleotide pair GRE1A/B is 82 nucleotides long and when annealed result in a DNA fragment flanked with a HindIII site at the 5' end and a Sall site at the 3' end:

GRE1A 5'agcttcgactgtacaggatgtttctagctactgtagtagctagaacatcctgtacagtgcagtcgagtacagtcgagtcgactacaacatcctgtacag 3'
GRE1B
5’cagctgtacaggatggtttcagctctcagctgatctcaggattctctagctactcagctgacgtgaagctgagctgacatccgta cagtcga 3’.

The second pair of oligonucleotides is flanked by a SalI site at the 5’ end and a BamHI site at the 3’ end
5 GRE2A 5’ tgcagctgtacagaaacatctcgtacagtctagctagctagaaatcctgt
acagtctgtagctgataacatctgctgtacag 3’
GRE2B 5’gatctgtacaggtgagtctactcagctactcaggtgtctactcagctactcaggtgtctactcagagttgtctagctag 3’.

The resulting plasmids were named p221.9GRE6 (Figure 13) and p221.10GRE6 (Figure 14)(used in later Example). The difference between p221.9 and p221.10 plasmids is that p221.9 contains the -60 35SCaMV minimal promoter while p221.10 (p221.10GRE6) contains the -46 35SCaMV minimal promoter.

Method

Protoplasts were isolated from a maize suspension culture derived from BE70 x A188 embryogenic callus material, which was maintained by subculturing twice weekly in MS0.5 medium (MS medium supplemented with 3% sucrose, 690mg/l proline, 1g/l myo-inositol, 0.2g/l casein acid hydrolysate, 0.5mg/l 2,4-D, pH5.6). Cells from suspensions two days post subculture were digested in enzyme mixture (2.0% Cellulase RS, 0.2% Pectolyase Y23, 0.5M Mannitol, 5mM CaCl2, 2H2O, 0.5% MES, pH5.6, ~660mmol/kg) using ~10ml/g cells, incubating at 25°C, dim light, rotating gently for ~2 hours. The digestion mixture was sieved sequentially through 250μm and 38μm sieves, and the filtrate centrifuged at 700rpm for 3.5 minutes, discarding the supernatant. The protoplasts were resuspended in wash buffer (0.358M KCl, 1.0mM NH4NO3, 5.0mM CaCl2, 2H2O, 0.5mM KH2PO4, pH4.8, ~670mmol/kg) and pelleted as before. This washing step was repeated. The pellet was resuspended in wash buffer and the protoplasts were counted. Transformation was achieved using a Polyethylene glycol method based on Negruțiu et.al. Protoplasts were resuspended at 2 x 10⁶/ml in MaMg medium (0.4M Mannitol, 15mM MgCl2, 0.1% MES, pH5.6, ~450mmol/kg) aliquoting 0.5ml / treatment (i.e. 1x10⁶ protoplasts / treatment). Samples were heat shocked at 45°C for 5 minutes then cooled to room temperature. 10μg each of p221.9GRE6 and pMF6HR1PAT (GR) (1mg/ml) / treatment were added and mixed in gently, followed by immediate addition of 0.5ml warm (-45°C) PEG solution (40% PEG 3,350MW in 0.4M Mannitol, 0.1M Ca(NO3)2, pH8.0), which was mixed in thoroughly but gently. Treatments were incubated at room temperature for 20-25 minutes, then 5ml 0.292M KCl (pH5.6, ~530mmol/kg) was added step-wise, 1ml at a time, with mixing. The treatments were incubated for a further 10-15 minutes prior to pelleting the protoplasts by centrifuging as before. Each protoplast treatment was resuspended in 1.5ml culture medium (MS medium, 2% sucrose, 2mg/l 2,4-D, 9% Mannitol, pH5.6, ~700mmol/kg) +/- 0.0001M dexamethasone (glucocorticoid). The samples were incubated in 3cm dishes at 25°C, dark, for 24-48 hours prior to harvesting. Fluorometric
assays for GUS activity were performed with the substrate 4-methylumbelliferyl-D-glucuronide using a Perkin-Elmer LS-35 fluorometer (Jefferson et al., 1987). Protein concentration of tissue homogenates were determined by the Bio-Rad protein assay (Bradford, 1976). The method was repeated for each effector construct.

Results

Reporter gene assay

A reporter gene construct (p221.9GRE6) was generated containing the GUS reporter gene under the control of a -60 CaMV 35S promoter with 6 copies of the glucocorticoid response element. To test this construct was functional in maize protoplasts a co-transformation assay was performed with the reporter construct p221.9GRE6 and the effector construct pMF6HR1PAT (GR) construct containing the entire glucocorticoid receptor.

Figure 15 shows that Reporter p221.9GRE6 alone or reporter plus effector pMF6HR1PAT (GR) with no activating chemical gave no significant expression. When reporter plus effector were co-transformed into maize protoplasts in the presence of 0.0001M dexamethasone (glucocorticoid), a significant elevation of marker gene activity was observed (Figure 15). The response is specific to glucocorticoid as the steroid Muristerone A does not lead to induced levels of expression. These studies clearly show the reporter gene construct p221.9GRE6 is capable of monitoring effector /ligand mediated gene expression.

Chimeric ecdysone effector constructs mediate inducible expression in maize transient protoplasts assays

A chimeric effector plasmid pMF6GREcRS was constructed, containing the ligand binding domain from the Drosophila ecdysone receptor and the DNA binding and transactivation domain from the glucocorticoid receptor. To confirm the reporter gene construct p221.9GRE6 could respond to a chimeric ecdysone effector construct, a series of co-transformation into maize protoplasts was performed.

Figure 16 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF6GREcRS) with no activating chemical, gave no significant expression in maize protoplasts. When reporter plus effector were co-transformed into maize protoplasts in the presence of 100μM Muristerone A, a significant elevation of marker gene activity was observed. The response was specific to Muristerone A, as the steroid dexamethasone did not lead to induced levels of expression. These studies clearly showed the reporter gene construct p221.9GRE6 is capable of monitoring chimeric ecdysone effector /ligand mediated gene expression.

A second chimeric effector construct pMF6GRHcR, was generated containing the ligand binding domain from Heliothis ecdysone receptor. When co-transformed into maize protoplasts with the reporter plasmid p221.9GRE6, no response to 100μM Muristerone or
100µM dexamethasone was observed (Figure 17). These data clearly show the Drosophila and Heliothis ligand binding domains exhibit different properties.

When the effector plasmid pMF6GREcRS, containing the ligand binding domain from Drosophila, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysonic agonists RH5849 and RH5992 (mimic), no chemical induced reporter gene activity was observed (Figures 18 and 19).

When the effector plasmid pMF6GRHcER, containing the ligand binding domain from Heliothis, was tested with the reporter p221.9GRE6 in presence of the non-steroidal ecdysonic agonists RH5992 (mimic), significant chemical induced reporter gene activity was observed (Figure 20). These data demonstrate the ligand binding domain from Heliothis has different properties to the Drosophila receptor in that the former responded to the non-steroidal ecdysteroid agonist RH5992. Figure 21 demonstrates the effector plasmid pMF6GRHcER confers RH5992 dependant inducibility on the reporter p221.9GRE6 in a dose responsive manner. Induction was observed in a range from 1µM-100µM RH5992.

Example IV - Testing of effector vectors in Tobacco protoplasts

The experiments carried out in the previous example demonstrated the specific effect of RH5992 (mimic) on pMF6GRHcER in maize protoplasts. It is the aim in this example to show the generic application to plants of the glucocorticoid/Heliothis ecdysonic chimeric receptor switch system. Tobacco shoot cultures cv. Samsun, were maintained on solidified MS medium + 3% sucrose in a controlled environment room (16 hour day / 8 hour night at 25°C, 55% R.H.), were used as the source material for protoplasts. Leaves were sliced parallel to the mid-rib, discarding any large veins and the slices were placed in CPW13M 13% Mannitol, pH5.6, ~860mmol/kg) for ~1 hour to pre-plasmolyse the cells. This solution was replaced with enzyme mixture (0.2% Cellulase R10, 0.05% Macerozyme R10 in CPW9M (CPW13M but 9% Mannitol), pH5.6, ~600mmol/kg) and incubated in the dark at 25°C overnight (~16 hours). Following digestion, the tissue was teased apart with forceps and any large undigested pieces were discarded. The enzyme mixture was passed through a 75µm sieve and the filtrate was centrifuged at 600rpm for 3.5 minutes, discarding the supernatant. The pellet was resuspended in 0.6M sucrose solution and centrifuged at 600rpm for 10 minutes. The floating layer of protoplasts was removed using a pasteur pipette and diluted with CPW9M (pH5.6, ~560mmol/kg). The protoplasts were again pelleted by centrifuging at 600rpm for 3.5 minutes, resuspended in CPW9M and counted. A modified version of the PEG-mediated transformation above was carried out. Protoplasts were resuspended at 2x10⁶/ml in MaMg medium and aliquotted using 200µl / treatment (i.e. 4x10⁵ protoplasts / treatment). 20µg each of pMF6GRHcRS and p221.9GRE6 DNA (1mg/ml) were added
followed by 200μl PEG solution and the solutions gently mixed. The protoplasts were left to incubate at room temperature for 10 minutes before addition of 5ml MSP19M medium (MS medium, 3% sucrose, 9% Mannitol, 2mg/l NAA, 0.5mg/l BAP, pH5.6, ~700mmol/kg) +/- 10 μM RH5992. Following gentle mixing, the protoplasts were cultured in their tubes, lying horizontally at 25°C, light. The protoplasts were harvested for the GUS assay after ~24 hours.

**Effector construct**

(i) Construction of a Dicotyledonous expression vector

The vector produced is a derivative of pMF6. pMF6GREcRS was restriction enzyme digested with PstI to produce 3 fragments namely, 3.4(Adh Intronless pMF6), 3.2(GREcRS) and 0.5(Adh intron I) kb. Isolation and religation of the 3.4 and 3.2 kb fragments resulted in pMF7GREcRS (Figure 22). pMF7GREcRS was restriction enzyme digested with EcoRI/SacI resulting in the 3.4kb pMF7 EcoRI/SacI vector which when isolated and purified was ligated to a 1.5 kb EcoRI/XhoI N-terminal end of the glucocorticoid receptor and the 1.2 kb Sall/SacI *Heliothis* ecdysone C-terminal end sequences to produce pMF7GRHEcR (Figure 23).

**Reporter plasmid**

The reporter plasmids constructed for the maize transient experiments were the same as those used without alteration in the tobacco leaf protoplast transient expression experiments.

**Results - Chimeric ecdysone effector constructs mediate inducible expression in tobacco transient protoplast assays**

Experiments were performed to demonstrate that the effector plasmid pMF6GRHEcR can confer chemical dependant inducible expression on the reporter p221.9GRE6 in tobacco mesophyll protoplasts.

Figure 24 shows that reporter (p221.9GRE6) alone or reporter plus effector (pMF7GRHEcR) with no activating chemical, gave no significant expression in tobacco protoplasts. When reporter plus effector were co-transformed into tobacco protoplasts in the presence of 10μM RH5992, a significant elevation of marker gene activity was observed. These data show a chimeric ecdysone effector construct, containing the *Heliothis* ligand binding domain can confer non-steroidal ecdysteroid dependant expression on reporter gene constructs in both monocotyledenous and dicotyledenous species.
Example V - Chimeric activity in Mammalian cells

Effector constructs

(i) Construction of Glucocorticoid\textit{Heliothis} ecdysone chimeric receptor.

The mammalian expression vector used in this experiment was pcDNA3 (Invitrogen). The GRHeR 2.7kb BamHI DNA fragment (isolated from pMF6GRHeR) was introduced into the pcDNA3 BamHI vector. The recombinants were oriented by restriction enzyme mapping. The DNA sequence of the junctions was determined to ensure correct orientation and insertion (pcDNA3GRHeR, Figure 25).

Reporter construct

The reporter plasmid for mammalian cell system was produced by taking pSWBGAL plasmid and replacing the CRESW SpeI/Clal fragment for a synthetic 105 bp DNA fragment containing 4 copies of the glucocorticoid response element (GRE) and flanked by SpeI at the 5' end and AflIII at the 3' end.

The oligonucleotides were synthesised using the sequences:

\text{GREspe1}

\begin{verbatim}
5'ctagtgtgtagagttgtcatccgctagtagcagctagaacatcctgtagtcagctagctagaacatcctgtagctagaacatcctgtcacac 3'
\end{verbatim}

\text{GREafl2}

\begin{verbatim}
5'ttaagttcatccgagttgctagactgtaggtttctactcgactgtagctagctagctagctagctagctagctagctagctagctagctagctgatcagc 3'.
\end{verbatim}

The two oligonucleotides were purified annealed and ligated to pSWBGAL SpeI/AflII to produce pSWGRE4 (Figure 26).

Results - Chimeric HECr drives transient reporter gene expression in mammalian cells

No expression was detected when a reporter gene construct pSWGRE4, comprising of a minimal \(\beta\)-globin promoter containing four copies of the glucocorticoid response element, fused to a \(\beta\)-galactosidase reporter gene, was introduced into CHO cells. Similarly, no expression was detected when pSWGRE4 and an effector plasmid pCDNA3GRHeR, containing the transactivation and DNA binding domain from the glucocorticoid receptor and the ligand binding domain from the \textit{Heliothis} ecdysone receptor, under the control of the CMV promoter were co-transformed into CHO-K1 or HEK293 cells. When co-transformed CHO (Figure 27) and HEK293 cells (Figure 28) were incubated in the presence of the non-steroidal ecdysone agonists RH5992 (mimic), significant chemical induced reporter gene activity was observed. Equally, induction of reporter activity was observed when HEK293 cells transfected with pcDNA3GRHeR and reporter were treated with Muristerone A (Figure 28).
Example VI - Screening system allows new chemical activators and modified ligand binding domains to be tested in Mammalian cells

The basis of a screening system are in place after the demonstration that the chimeric receptor was activated in the presence of RH5992. A screen was carried out using CHO cells transiently transfected with both pSWGRE4 (reporter) and pcDNA3GRHecR (effector) constructs. In the first instance 20 derivatives compounds of RH5992 were screened. It was observed that 7 out of the 20 compounds gave an increased reporter gene activity compared to untreated cells. A second screen was carried out in which 1000 randomly selected compounds were applied to transiently transfected CHO cells. Two compounds were found to activate reporter gene activity above that from the untreated controls. The second screen suggest that this cell based assay is a robust and rapid way to screen a small library of compounds, where a thousand compounds can be put through per week.

Example V - Stably transformed Tobacco plants

Stable Tobacco vectors

The components of the stable Tobacco vectors were put together in pBluescript prior to transfer into the binary vector. The production of stable transformed plants entails the production of a vector in which both components of the switch system (ie. effector and reporter) are placed in the same construct to then introduce into plants.

The methodology described below was used to produce four different stable Tobacco vectors. The method involves three steps:

1. pBluescript SK HindIII/EcoRI vector was ligated to either GRE6-4635SCaMVGUSNOS HindIII/EcoRI (from p221.10GRE6) or GRE6-6035SCaMVGUSNOS HindIII/EcoRI (from p221.9GRE6) resulting in plasmid pSK-46 and pSK-60.

2. This step involves the addition of the chimeric receptor (35SGRHEcRNOS or 35SGRVP16HEcRNOS) to pSK-60 or pSK-46. Thus a pSK-60 (or pSK-46) Xbal vector was ligated with either the 3.4kb 35SGRHEcRNOS Xbal or the 3.0kb 35SGRVP16HEcRNOS Xbal DNA fragment to produce pSKES1 (pSKGRE6-6035SCaMVGUSNOS-35SGRHEcRNOS), pSKES2 (pSKGRE6-4635SCaMVGUSNOS-35SGRHEcRNOS), pSKES3 (pSKGRE6-6035SCaMVGUSNOS-35SGRVP16HEcRNOS) and pSKES4 (pSKGRE6-4635SCaMVGUSNOS-35SGRVP16HEcRNOS).
3. Transfer from pBluescript based vectors to binary vectors. The transfer of the ES1 (Figure 29) ES2 (Figure 30), ES3 (Figure 31) or ES4 (Figure 32) DNA fragments into the binary vector JR1 involves five steps:

(i) Restriction enzyme digestion of pSKES1 (ES2, ES3, and ES4) with ApaI and NolI to liberate the insert from the vector pBluescript.

(ii) The two DNA fragments were BamHI methylated for 2 hours at 37ºC in TRIS-HCl, MgCl₂, 80uM SAM (S-adenosylmethionine) and 20 U of BamHI methylase.

(iii) Ligate a ApaI/NolI linker onto the fragment. The linker was designed to have an internal BamHI site:

```
ApaBNot1 5’ cattggatctttagc 3’ and
ApaBNot2 5’ggccgcttaagatccaatgggcc 3’.
```

(iv) Restriction enzyme digest the protected and linked fragment with BamHI and fractionate the products on a 1%(w/v) Agarose gel. The protected DNA fragment (5.5kb) was cut out of the gel and purified.

(v) A ligation of JR1 BamHI vector with the protected band was carried out to produce JRIESI (JRIES2, JRIES3 or JRIES4). The DNA of the recombinant was characterised by restriction mapping and the sequence of the junctions determined.

The plant transformation construct pES1, containing a chimeric ecdysone receptor and a reporter gene cassette, was transferred into Agrobacterium tumefaciens LBA4404 using the freeze/thaw method described by Holsters et al. (1978). Tobacco (Nicotiana tabacum cv Samsun) transformants were produced by the leaf disc method (Bevan, 1984). Shoots were regenerated on medium containing 100mg/l kanamycin. After rooting, plantlets were transferred to the glasshouse and grown under 16 hour light/8 hour dark conditions.

**Results - Chimeric ecdysone effector constructs mediate inducible expression in stably tobacco plants**

Transgenic tobacco plants were treated in cell culture by adding 100µM RH5992 to MS media. In addition seedlings were grown hydroponically in the presence or absence of RH5992. In further experiments 5mM RH5992 was applied in a foliar application to 8 week old greenhouse grown tobacco plants. In the three methods described uninduced levels of GUS activity were comparable to a wild type control, while RH5992 levels were significantly elevated.
Ecdysone switch modulation and optimisation

Example VIII - Yeast indicator strains for primary screen of chemical libraries

A set of yeast indicator strains was produced to use as a primary screen to find chemicals which may be used in the gene switch. The properties of the desired chemicals should include high affinity resulting in high activation but with different physico-chemical characteristics so as to increase the scope of application of the technology. Moreover, the production of this strain also demonstrates the generic features of this switch system.

Effector vector

A base vector for yeast YCp15Gal-TEV-112 was generated containing:
Backbone - a modified version of pRS315 (Sikorski and Hieter (1989) Genetics 122, 19-27)-a shuttle vector with the LEU2 selectable marker for use in yeast;
ADH1 promoter (BamHI- Hind III fragment) and ADH1 terminator (Not I- Bam HI fragment) from pADNS (Colicelli et al PNAS 86, 3599-3603);
DNA binding domain of GAL4 (amino acids 1-147; GAL4 sequence is Laughon and Gesteland 91984) Mol. Cell Biol. 4, 260-267) from pSG424 (Sadowski and Ptashne (1989) Nuc. Acids Res. 17, 7539);

The plasmid contains unique Eco RI, Nco I and Xba I sites between the DNA binding domain and activation domains.

Into this vector a PCR DNA fragment of the Heli othis ecdysone receptor containing the hinge, ligand binding domains and the C-terminal end was inserted. The 5' oligonucleotide is flanked by an NcoI restriction recognition site and begins at amino acid 259:

HecrNcoI 5' aattcatgtctagacagcatagcagcatagcagc 3'.

The 3' oligonucleotide is flanked by an XbaI site and encodes for up to amino acid

HecRXbaI 5' ctgaggtctagacagctgccggcggc 3'.

The PCR was carried out using vent polymerase with the conditions described in Example IA. The fragment was restriction enzyme digested with NcoI and XbaI purified and ligated into YCp15GALTEV112 NcoI/XbaI vector to produce YGALHeCRB112 or TEV-B112 (Figure 34). In order to reduce constitutive activity of the YGALHeCRB112 plasmid a YGALHeCR plasmid was produced in which the B112 activator was deleted by restriction enzyme digesting YGALHeCRB112 with XbaI/SpeI followed by ligation of the resulting
vector (i.e. SpeI and XbaI sites when digested produce compatible ends) (TEV-8, Figure 33). An effector plasmid was constructed whereby the B112 transactivating domain was excised from YGalHecRB112 with XbaI and replaced with the VP16 transactivation domain DNA fragment (encoding amino acids 411 and 490 including the stop codon). The resulting vector was named YGalHecRVP16 or TEVVP16-3 (Figure 35).

**Reporter construction for yeast**

The *S. cerevisiae* strain GGY1::171 (Gill and Ptashne (1987) Cell 51, 121-126), YT6::171 (Himmelfarb et al (1990) Cell 63, 1299-1309) both contain reporter plasmids consisting of the GAL4-responsive GAL1 promoter driving the E. coli B-galactosidase gene. These plasmids are integrated at the URA3 locus. The reporter strain YT6::185 contains the reporter plasmid pJP185 (two synthetic GAL4 sites driving the B-galactosidase gene) integrated at the URA3 locus of YT6 (Himmelfarb et al). (Note- the parental strains YT6 and GGY1 have mutations in the GAL4 and GAL80 genes, so the reporter genes are inactive in the absence of any plasmids expressing GAL4 fusions).

**Yeast assay**


**Results - Automated screening system allows new chemical activators and modified ligand binding domains to be tested in yeast**

An effector vector pYGalHecRB112 has been generated containing a GAL4 DNA binding domain, a B112 activation domain and the ligand binding region from *Heliothis virescens*. In combination with a GAL reporter vector, pYGalHecRB112 form the basis of a rapid, high throughput assay which is cheap to run. This cell-based assay in yeast (*Saccharomyces cerevisiae*) will be used to screen for novel non-steroidal ecdysone agonists which may of commercial interest as novel insecticides or potent activators of the ecdysone gene switch system. The demonstration of an efficient system to control gene expression in a chemical dependant manner, forms the basis of an inducible system for peptide production in yeast.

The yeast screening system forms the basis of a screen for enhanced ligand binding using the lac Z reporter gene vector to quantitatively assay the contribution of mutation in the ligand binding domain. Alternatively, enhanced ligand binding capabilities or with a selection cassette where the lac Z reporter is replaced with a selectable marker such as uracil (URA 3), tryptophan (Trp1) or leucine (Leu2), and histidine (His). Constructs based on
pY GAL HEcRB112 with alterations in the ligand binding domain are grown under selection conditions which impair growth of yeast containing the wild type ligand binding domain. Those surviving in the presence of inducer are retested and then sequenced to identify the mutation conferring resistance.

Example IX - Optimisation of chimeric receptor using a strong transactivator

Construction of mammalian expression plasmid with chimeric receptor containing Herpes Simplex VP16 protein sequences.

The construction of this chimeric receptor is based on replacing the sequences encoding for the glucocorticoid receptor transactivating domain with those belonging to the VP16 protein of Herpes simplex. Thus PCR was used to generate three fragments all to be assembled to produce the chimeric receptor. The PCRs were carried out as described in Example II, iii. The first fragment includes the Kozak sequences and methionine start site of the glucocorticoid receptor to amino acid 152 of the glucocorticoid receptor. The oligonucleotides used for the generation of this fragment included an EcoRI site at the 5’ end: GR1A 5’ atataaatccacacctgactccaaagaat 3’ and at the 3’ end a NheI restriction enzyme recognition site:
GR1B 5’ atatctagctgtgggggacagacacacagctgg 3’.

The second fragment also belongs to the glucocorticoid receptor and begins with a NheI site in frame with amino acid 406:
GR2A 5’atatctagctctcagctctcaacagcacaac 3’ and ends with a XhoI site at amino acid 500:
GR2B 5’ataatcggagcatacctttatatatat 3’.

The two fragments were introduced into pSKEcoRI/SacI in a ligation containing GR1A/B EcoRI/NheI, GR2A/B NheI/XhoI and HEcR SalI/SacI (from pSKHEcRDEF) to yield pSKGRDHEcR. The GR sequences and junctions of the ligation were found to be mutation free.

The third fragment to be amplified was a sequence between amino acid 411 to 490 of the herpes simplex VP16 protein. The amplified fragment was flanked with SpeI recognition sites. SpeI produces compatible ends to those of NheI sites. The oligonucleotides used:
VP16C 5’ attactagttctgggcccctccgacgat 3’ and
VP16E 5’ attataagtccccacgactctgcagc 3’
produced a 180 bp fragment which was restriction enzyme digested with SpeI and introduced into pSKGRΔHEcR NheI vector to produce pSKGRVP16HEcR. The DNA from the latter was sequenced and and found to be mutation free, the junctions were also shown to be in frame with those of the glucocorticoid receptor.
The 2.2 kb EcoRV/NotI GRVP16HEcR fragment was introduced into a pcDNA3 EcoRV/NotI vector resulting in pcDNA3GRVP16HEcR (Figure 36).

Construction of plant transient expression effector plasmids containing the chimeric receptor with VP16 sequences

The same procedure was carried out to clone the GRVP16HeCR DNA fragment into tobacco(pMF7b) and maize(pMF6) expression vectors. A 2.2kb BamHI DNA fragment was isolated from pcDNA3GRVP16HeCR and ligated into the pMF6 BamHI (or pMF7b BamHI) vector to produce pMF6GRVP16HeCR (Figure 37) (or pMF7GRVP16HeCR) (Figure 38).

Results - Addition of strong activation domains enhance ecdysone switch system

The VP16 transactivation domain from herpes simplex virus has been added to a maize protoplast vector pMF6GRHecR to generate the vector pMF6GRVP16HeCR. When co-transformed into maize protoplasts with the reporter construct p221.9GRE6, in the presence of 100μM RH5992, enhanced levels of expression were seen over pMF6GRHecR. Figure 39, shows that RH5992 is able to induce GUS levels comparable to those observed with the positive control (p35SCaMVGUS), moreover, a dose response effect is observable.

VP16 enhanced vectors (pES3 and pES4) have been generated for stable transformation of tobacco. Following transformation transgenic progeny containing pES3 and pES4, gave elevated GUS levels following treatment with RH5992, relative to comparable transgenic plants containing the non-VP16 enhanced vectors pES1 and pES2.

An enhanced mammalian vector pcDNA3GRVP16HEcR was prepared for transient transfection of mammalian cell lines. Elevated reporter gene activities were obtained relative to the effector construct (pCDNA3GRHEcR) without the VP16 addition.

"Acidic" activation domains are apparently "universal" activators in eukaryotes (Ptashne (1988) Nature 335 683-689). Other suitable acidic activation domains which have been used in fusions are the activator regions of GAL4 itself (region I and region II; Ma and Ptashne (Cell 1987) 48, 847-853), the yeast activator GCN4 (Hope and Stuhl (1986) Cell 46, 885-894) and the herpes simplex virus VP16 protein (Triezenberg et al (1988) Genes Dev. 2, 718-729 and 730-742).

Other acidic and non-acidic transcriptional enhancer sequences for example from plant fungal and mammalian species can be added to the chimeric ecdysone receptor to enhance induced levels of gene expression.

Chimeric or synthetic activation domains can be generated to enhance induced levels of gene expression.
Example X - Optimisation by replacement of Heliothis ligand binding domain in chimeric effector for that of an ecdysone ligand binding domain of another species

Mutagenesis of the ecdysone ligand binding domain results in the increased sensitivity of the chimeric receptor for activating chemical. This can be achieved by deletions in the ligand binding domain, use of error prone PCR (Caldwell et al., PCR Meth. Applic 2, 28-33 1992), and in vitro DNA shuffling PCR (Stemmer, Nature 370, 389-391 1994). To enhance the efficacy of the listed techniques we have developed a screening system for enhanced levels of induced expression (see below).

An alternative strategy to the mutation of a known ligand binding domain is to identify insect species which are particularly sensitive to ecdysteroid agonists. For example Spodoptera exigua is particularly sensitive to RH 5992. To investigate the role of the ecdysone receptor ligand binding domain in increased sensitivity to RH5992 we have isolated corresponding DNA sequences from of S. exigua (Figure 40, Sequence ID No. 6). Figure 41, Sequence ID No. 7 shows a protein alignment of the hinge and ligand binding domains of the Heliothis virescens and Spodoptera exigua ecdysone receptors. The protein sequence between the two species is well conserved.

Results - Manipulation of the ligand binding domain leads to enhanced induced expression

Isolation of an ecdysone ligand binding domain from another lepidopteran species was carried out by using degenerate oligonucleotides and PCR of first strand cDNA (Perkin Elmer, cDNA synthesis Kit) of the chosen species. The degenerate oligonucleotides at the 5' end were HingxhoA and B and at the 3' end ligandxA/B

HingxhoA 5' attgctcagaaagicgcagagtgcggttgtgta 3'  
HingxhoB 5' attgctcagaaagicgcagagtgcggttgtgta 3'  
ligandxA 5' ttactcgacgtccccaiatctctctcagaa 3'  
ligandxB 5' ttactcgacgtccccaiatctctctcagaa 3'

RNA was extracted from 4th instar larvae of Spodoptera exigua since Spodoptera exigua appears to be more sensitive to RH5992 than Heliothis (Smagghe and Degheele,
1994). The first strand cDNA was used in PCR reactions under the following conditions
20mM Tris-HCl (pH8.4), 50mM KCl, 1.5mM MgCl2, 200mM dNTPs (dTTP) and 0.02 U/ml Taq DNA polymerase and in the presence of 1ug of each Hinge (5' 3') and Ligand (5'3') oligonucleotides. The PCR cycling conditions were 94°C for 1 minute,
60°C for 2 minutes and 72°C for 1 minute and 35 cycles were carried out. A sample of the
completed reaction was fractionated in a 1% agarose (w/v) 1 x TBE gel, and the resulting 900
bp fragment was subcloned into pCRII vector (Invitrogen). The resulting clone (pSKSeC 1-
10) were further characterised and sequenced.

Example X - Manipulation of reporter gene promoter regions can modulate chemical
induced expression

The context of the effector response element in the reporter gene promoter can be
used to modulate the basal and induced levels of gene expression. Six copies of the
glucocorticoid response element were fused to 46 bp or 60 bp of the CaMV 35S promoter
sequence. When used with the effector construct pMF7GRHEcRS the reporter gene
construct containing 46 bp of the CaMV 35S promoter gave reduced basal and induced levels
of GUS expression relative to the 60 bp reporter construct (Figure 42).

Constructs for plant transformation (pES1 and ES2) have been generated to
demonstrate the size of minimal promoter can be used to modulate the basal and induced
levels of gene expression in plants.

The number and spacing of response elements in the reporter gene promoter can be
adjusted to enhance induced levels of trans-gene expression.

The utility of a two component system (effector and reporter gene cassettes) allows
the spatial control of induced expression. Trans-gene expression can be regulated in an tissue
specific, organ specific or developmentally controlled manner. This can be achieved by
driving the effector construct from a spatially or temporally regulated promoter.

References
Allan, G.F., Leng, X., Tsai, S.Y., Weigel, N.L., Edwards, D.P., Tsai, M.-J. and O'Malley,
Carlberg, C., Bendik, I., Wyss, A., Meier, E., Sturzenbecker, L.J., Grippo, J.F. and Hunziker,
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ZENECA LIMITED
(B) STREET: 15 STANHOPE GATE
(C) CITY: LONDON
(E) COUNTRY: UK
(F) POSTAL CODE (ZIP): W1Y 6LN

(ii) TITLE OF INVENTION: A GENE SWITCH

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
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(B) FILING DATE: 07-JUL-1995

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(B) FILING DATE: 24-AUG-1995

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9605656.9
(B) FILING DATE: 18-MAR-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Heliothis virescens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
TGCGAGGGGT GCAAGGAGIT CTTCAAGCCG AGTGTAAAACAAAATGCAGT GTACATATGC
AAATTCGGCC ATGCCTGGCG AATGGAATATG TATATGCCGA GAAATGCGCA AGAGTA

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1934 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Heliothis virescens

(vii) IMMEDIATE SOURCE:
(B) CLONE: pSK19R

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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TCCACTGGTG TTTTACCAC CACAGAAAAAG GCCCTCTGCTC ATTTAGAGGG TGGTGCCTAG 60
AAGGTCACTCA TTCTCTGCTG CCCACGCCGTG ACCCATGTTC GTCTTGGGTG TCAACCTTGAA 120
AGCAGATAGCC CCCCTTTCAC AAGGTCACTTC CCAACGCCCT CTCGACCAACC AACTGCGCTG 180
CTCCCTCTGCC TAAGTGCTAC CATGACAACCT TCGAGATCAT TGAAGGTCTG ATGACCACTG 240
TACACGCCAC CACTGGCCACC CAGAAGACAG TGATGGAAC CTCCTGTTAAA CGTGGCCGGT 300
ATGCAGCGTTG TGCTCAGACC AAATATCATTC CCCGCGAATTT CCCAGGCAGC AGCTAGCTAA 360
CCTGCGACGAC ACAAACCCCT TACCTTCCTA TCCGTATCCA ATGCCACCCA GAACACCCCA 420
ATCAGAAAC CAGTCAATGT CATCAGCTGC TGGAGAATCT GCTCCAGCTT CGATGTTAAA 480
CGGCTGCAAGT ACAGATGGGC AGGCGAGGCG GCAGAAGAAA GCCGCGACGC GCAGACAGCA 540
AGAAGAGCTA TGCTCTGGCT GCCGCCAGAC AGCCTCCGGA TACACTACA AGCGCGCTCAG 600
ATGTGAAGGG TGTTAGGGTT TTCTCAGGCG GAGTGTAAACC AAAAAATGCG AGTACAAATG 660
CAGTCTGCGC CACAGTGGGA AAATGGAATCT ATATATGGGC AGAAAAATGCG AGGAGTGGTGTG 720
GTGAGAGAAA TGCTCTGGCG TGCCGATGGC GCGCGATGCG GTGTGCGCGC AGAACAGAGT 780
TGGCAGAAA AACAGGGAA AAAAAAGCGCA GAGGAAAAAA GACAAAATGC CCGTCAGTAC 840
AGAGAGTGA GACATCTCA TGCTCCCTAC CATGCAATGT GACCCTCCGC CCCGGAGGCC 900
CGCTAGAAAT TCAGAATGTC TGGACAGCAA GGTGGTTGCA CGATCTCTGA ATGGAGACGT 960
AATGGAACAG AACAGATGGA AGAAGCTGCG GCCATCCACT GCCAAATCA AGATGTTGTAT 1020
CCGAAAGGTC GCTGGTGGAAG AAGAGGCTG TGAACCAACCT TCGGAGGAAG ACCTGAAGAG 1080
GGCTACAGC TCGACAGAGG ACGACGAAATC ATGCAGCTGC CCGTCCGCTG AGATTTACGGA 1140
GATGACGGAC TCACAGCTGC AGCTCATCAAG AAATCCCGCT CCGGCTCTCC GCAGGCTCGC 1200
CAAGATCTGC CAGTCCAGAC ATGACAGTTA ATTAAGGGCG TGCTCAAGTG AGTGTGATATG 1260
GCTCCAGAGG GCGCCGCCAT ATGACGCGCG CACCGACAGC GTACTGCCTCG CAGAACAACCA 1320
GGCCGTACACT CGCGACAACCT AGCGCAAGG GCGATGCGCG TACGTCATCG AGGACCTGCT 1380
```
GCACCCTCTGT CGGTGACATGT ACTCCATGAT GATGGAATAAC GTGCATTTATG CGCTGCTTAC 1440
AGCCATTGTC ATCTTCTCAG ACCGCGCGCC GCTTGAGCAA CCCCCTTGGG TGGAGGACAT 1500
CCAGAGATAT TACCTGAACA CGCTACGGGT GTAATACTCTG AAACAGAAACA GCGGGTCCGC 1560
CCGGCGGCCT GCTATCTTCCG GCGAGATCTT GGAGCATACTG AGCGAGATCC GCACGCTTGG 1620
CATGCAGAAC TCCAACATGT GCATCTCCCT CAAGCTGAGA AAACAGAAACG TCACCGCCCCTT 1680
CTCTGAGGGAG ATCTGGGAGC TGGCCGACGG ACGGACAGCG GGGCCACCGG TGGGGCGCGA 1740
GCCGGCGCGC CCTCTAGCCC CCGCGCGCGCC GGGCGCGCCG CCGCCACCGC TCTAGGCGCC 1800
CTCAGGGAGG AGAGCCCTCA AACGCCCGTC TTTTATGAAA GACGACCCCG ACTGACCTCG 1860
AGCTGATCAA CCTATTTTATA AGGACTGCAG ATTATACCAC TAAAAGGGAC ACACCCGATTAC 1920
CCGATTTCGT ACGG 1934

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2464 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINIAL SOURCE:
(A) ORGANISM: Heliothis virescens

(vii) IMMEDIATE SOURCE:
(B) CLONE: pSk16.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
CGCTGTTATA ACAACGGACC ATTCCAGACG CTGCGAATGC TCGAGGAGAG CTCGTCTTGAG 60
GTGACGCTGT CTCACGACT GGGCTCGGCC CCAGCTATGG TGTAGGCCC GGAATCGCTC 120
GCCGCGCGCC AGATCGGGCC CCTGGAGCTG TGGGCTACGG ACGATGCGCAT CACTTACAGC 180
ATGGCAAGCT CGTGGCGCAC TCGGACCATG GAGCAGCCAG AGGCCGCCGC GCAGCAGCAG 240
CCGGGACGAA CAGAACCCCT ACCTCTCATG CCGTTTACAA TGGGCCGCAAC GAAACCCAAA 300
TCAGAAAAAG AGTCAATGTC ATCAGGTGCTG GGGGAACGTG CTCCAGGCTTC GAGTTAAAC 360
GGCTGACGCA CAGATGCGCA GGGCGGCGGC CAGGAAGGAG GCCAGCGGCC GAGGCAGCAA 420
GAAGAGCATG GTCTTCTGCT GGCGGACAGA GCCCTCCGAG ATCACTACA CGCGCTCACA 480
TGTGAAAGGT GTAAAGGTTT CTTCCAGCGCC AGTGTAACCA AAAATCGAGT GTACATATTGC 540
AAATGCGGCC ATGCTTGCGA AAATTGATAC TATATGGGGA GAAAATGCTA GAGGCTGCGG 600
TTGAAAGAAA GTCTTGCGGT GGCGACAGAG CCGCAGCGCC TGGGCACGG AAGCAAGGTT 660
GCAATGAAAC GCAAAGAGA AAAGGGCCAG AGGGAAAAAG ACAAATGCTGC CGTCAGTACG 720
ACGACAGTAG ACAGACCATC GCCTCCCATC ATGCAATGTG ACCCTCCGCC CCCAGAGGCC 780
GCTAGAAATTG TGAATTGTGT GCACGACGAG GTGGTGGCCAC GATTTCTGAAA TGAGAAGCTA 840
ATGGAACAGA ACAGATTGAA GAACGTCGCA CCCCCTCATG CCAATCAAGA GTCGTTGATC 900
GCAAGGCTCG TGTTGTACCA GAAAAGCTAT GAACACCTTG CCGAGAAGA CCTGAAGAGG 960
TTTACACAGG CGAGACGAGA CGACGAAGAC TCGGATATGC CTTCCGCTCA GATTACCGAG 1020
ATGACGATTC TCACAGTGCA GCTCATCGTA GAATTCGCTA AGGGGCTCCC GGCTCTGCGC 1080
AAGATCCTCG AGTCGGACCA GATCCAGTTA TTTAAGGGCG TCTCAAGTGA GGTGATGATG 1140
CTCGAGGTTG CTCGGCGCTA TGACGCCGGC ACCGACGACG TACTGTTGCG GAACAACCAG 1200
GGCTACACTC GCGACACTCA CGCAAGAGCA GCCATGGCGT AGCTCATTGA GGACCTGCTG 1260
CATTCTGTTC GTGCGATGTA CTCTCAATGTG ATGGATAACG TGCAATTAGC GCTGGTTACA 1320
GCCATGTCGA CTTTCTCCGA CGGGCCGCGG CTGGAGCAA CCGTTGTTGT GGAGAGACATC 1380
CAGAGATATT ACTTGAACAA GCTACGATCG TACATCCCTG ACCAGAAGCG CGCGCTGGCC 1440
CGCGCGCGCG CTCATCTCGG CGAGATCTGG GCACATTGCA GCAGAGTCCG CACGCGTGGC 1500
ATGCAAGACT CCAACATGTT CATTCCCCTT AACGCTGAGA AGCAGGAAGT GCGCCGCTTC 1560
CTCGAGGAGA TCTGGAGCGT GCAGCAACGG TGAGACGACG TGACGCCTGG TGGCGCGCGG 1620
GGCGCGCGCG CTCTACCGCC CGCGCGCCGC GCCGCCGCGG CCAGGACCCT CTAGCGCGCC 1680
TCAGGAGAGA AGCCTCAGAT ACTGGCTAGT TTDTAGTGAAG TGCAAGCGCA CTGAGCTGCA 1740
CTGATGCAAC CTATTTAATA GGACTGCGAA TTDBAAGCTG TAAAGGGCA CACCGCTACC 1800
CGATTTCCGA CGATTTCCGA AGCCGAGAG ACTGGCGAGG TGACTGTTAG TGAAAATATATG 1860
CGGTGGTGA GAAGTTGAGA AAGTTAGTAT TTATGCGCTG TCTGGCGCGG CACCGCTAGG 1920
CCGCGCGCGC GCAGCGGCGG CGCGCGCGCG GCTAGGTTTTA TTTGCGTTTAC GACGTGGATTG 1980
GTCACCCGGA TACGACTGTA TGATAGACTG CCGTCGATAT AGTACACCTCT AAATATATAC 2040
CATAGTACG TAGCTTACGA GAGTTATGGA AGACAAAGGA TATAAGAAGA AGATGTGTCT 2100
ATTGGGGAGA AAGTTGATAG TTATGTTATG TTACAAAAAT TAACAAATAA AGCTTGGATTA 2160
ACCTTTCCGA TATAATATTG TGATGATGAT CGCGCTGCCGG CACGCGCTGG 2220
TTTCTGATAG AGCAGTGGAG NCGCTTTGCG TGTTTTATCG TTCCACTGGC CTGGCGCGCC 2280
GACCCCTGCA TAAATGAGTA ATTTAATTTA TGTCTGTGAT TACATTAAAA TGTGTGATT 2340
ATCTACCATG AGGCTGATAG ACTGTGGCTTT ATTACAAATAC AAGTGTGTGG TCGTCGATAG 2400
CTTCACACGC AGCAAGCCCTT TTTGTTAAGTG GATTACTGGA CATGGACACT CGACCCGAAA 2460
CTTC 2464
(2) INFORMATION FOR SEQ ID NO: 4:
WO 96/37609

- 44 -

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2745 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 225..1955
(D) OTHER INFORMATION: /codon_start= 225
/product= "Heliothis ecdysone receptor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ACTCGCGCTGC TCTTCTCACCC TGGTGCTCGG ATTTGTTGTTG ACTAGAAAAA AGTTGTCGCC 60
GCTCGAAAGA GACTTCCCGG TCCTATTGGAA TGGCAGCAGA GTGAGAGCCG TGGATAGCGA 120
TTGCATTTCG TTTGACATCT TCCCTGAGTG TGGTGTAGCT CAGACAGTCC GGTGGTACAT 180
AGTTTAAAGC GAGAGAAATA TGAAGTGAAA GCTTCTCTCG GAGGATGTCC TCTCGCGCTC 240
GGTGGATCGC GAGGAGTGAC ACCTGCGCGC ACACTAGACG CCGCTGATAT AACAAGGCCG 300
CATTCAGACG TGCAGGAAAG TCCTGCTGAT GGTGACGCTG TCTTCAGACG 360
TGGGCTGCTG GCCGCTATGC GTGATGTCGG CAGGAATCGCT CGCTGCGGCC GAGATGGCCG 420
GCCTGAGCT GTGGGGTCAC GACGATGCGA TCACCTACGA CATGAGACAG TGCTGGCCGA 480
CCCTGACCAT GGAGCGAGCG CAGCCCCAGC CGCCGACGCA GCCTGAGCCG ACACAACCCC 540
TACCCTCAGG AGCCCTGACG AGAGCGCGCG CAGCTTGCAG GAGTCAAATGG 600
CATCAGCTGCG TCGAGAACTG TCCTCCAGCTT CAGTGTAGAA CGGTGCTGAC ACAAGTGCAG 660
AGGCCGACGC GCAGAAAGAG GGGCCAGGCC CGAGCGAGCA AGAACGCTG TACGCTTCGT 720
GGCGGCCACG AGCTTCCCGG TATCACTACA ACCGCTCGAC ATGTTGCAGG TTTAAAGGTT 780
TCTTCAGGGG GAGGTGAACC AAAATGTCAG TGTCATACAG CAAATTCCCAG CTGCTTGCAG 840
AAATGGAAT ATATGCGCG AGGAAATGTC AGGAGTCGTC GGGAGGAAGA TGGTTAGCGG 900
TGCCGATGAG GCAGGCTGCG GCGTGCTGCCG AGAACAGCGT TGCAATGAA CGAAGAGAGA 960
AAAGGCCGCA GAGGAGAAA GACAAATGCG CGCTCAATGAC GACAGACGT GACACACCA 1020
TGCTCTCCTC CATGCAAGTGC ACAGCTGCCG CCCAGAGGCG CTAGATGAAAT TGGGAATGAG 1080
TGCGACGGAC GGTGGTGCA CAGTTCCTGA ATGAGAAGCT ATGGGAAGCG AACAGATGGA 1140
AGAACGCGCC CCCCTCCTTA GCCAACAGTGA AGCTGTGTCG TGGAAGCTTACC 1200
AAGAAGCTTA TGGACACCCG TCGAGGAGCG ATCTGAGAGG GTTTACAGCG TCGGAGGAGG 1260
AGCGAGGAGA CTGAGATATG CGCTGCTGTCG AGATCGACGA GATGACGATT CTCAACATGC 1320
AGCTCATGCT AGAATTCGCT AAGGGCTCCG CGGCTCTCCG CAAGATCTCG CAGTGCGACC 1380
AGATCAGTGT ATTAAAGGGC TGCTCAAGTG AGGTGATGAT GCTCCGAGTG GCTCGCGGCTT 1440
AGTACGCGGC CACCGACACG GTAAGTGTGCT CGAACAACCA GCGCTACACT CGCGCAACCT 1500
ACCGCAAGGC AGGCGTGGGC TACGTCTACG AGGACTCTGT GCACTTCTGT CGGTGCTAGT 1560
ACTCCATGAT GATGGAATAA GTGTACTTATG CGCTGCTTAC AGCATTGAAC ATCTTTCTAG 1620
ACCGGCCCAG CTTTGAGCAA CCCCCTGTTGG TGGAGGAGAT CGAGAGATAA TACCTGAACA 1680
CCCTAGGGTC GTACATCCTG AACCAGAACG GCGGTCGCGC CGCGGGCGCG GTCTCCTTCG 1740
GGCAGATCTC GGGCACTAGC ACGGAGATCC GCGCCCTGGG CAGTCGAGAC TCCACGATG 1800
GCATCTCCCT CAAGCGTAGA AACAGGAAAC TGCCGGCTTT CCTCGAGGAG ATCTGGGACG 1860
TGGCGGACGT GGGCGGACAG CGCGCGCGCG TGCCGGGGGA GCGCGCGCGC CCTCTAGCCC 1920
CCGCCGCCGC CGCAGCGCGCC CCGCCCGCGC TCTAGCGCGC TCTACGGAGAG AAGCTGACTA 1980
GACTGGCTAG TTTTAGTGAAG GTACACGAGAC ACTGAGCTGC AGCTGATCAG CTTATTATTA 2040
AGGACTGCGA ATTITACCAC TTAAGAGGCG ACAAGCGCTAC CGGATTTTG TAGTATTCCG 2100
TGACCCGAGA CGATGCAGAG GTGCTGTGTTAAT TGTAATATAT TTGTTGTGTA ACGATTAGG 2160
GAATATATAT TTGGTTGTGCT GTTCCGCCCC GCACCGCGTTC GCGGTCGCGC GCGATGGCGC 2220
GCCGCCGGCG CGGCTAGTGTTA ATTTCTGTTA CGACTGAGTT GTGCACCTGCG ATACGACTGT 2280
AGTATAAGAC TTTCTTCTGAT AGGTACACCT ACTAAATTAC ACATACGTAC GTAGCTTACG 2340
AGAGTTATTA GAGACAAGAG ATATAAGAAG AAGATGTGTTC TATTGGGTGA AAGTTTGATA 2400
GTTAGTTAAT TTTCAAAAAA TTAACAAATA TACGTTAGT AACCTCCGAG GTATAATATT 2460
GTGATGAGTCT GTCCCGCTGC CAGTCTCCGCG TCCACATTTTG GTTTTGCTATG CACACGCTG 2520
GNCGGTTATC GTGGTCTAGT GTCCCATACGT CTTGTCGCCG CGACCCCTCGA CTAATAGGAT 2580
AAATTTATTG ATCTGCTGGA TTACTATTTT ATGTTGCTAT TATCTACATG AGGGTGATAT 2640
AGATGTTCTT TTTACTAATA CAAAGTTTTG GTGCTGCGATA GTCTCCACAC GAGCAAGCT 2700
TTGTTTAAAG TGAATTTACTG ACATGACGAC TCGACCCCGA ACTTC 2745

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 575 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
Leu Lys Arg Val Thr Gln Ser Asp Glu Asp Asp Glu Asp Ser Asp Met
340  345  350

Pro Phe Arg Gln Ile Thr Glu Met Thr Ile Leu Thr Val Gin Leu Ile
355  360  365

Val Glu Phe Ala Lys Gly Leu Pro Gly Phe Ala Lys Ile Ser Gin Ser
370  375  380

Asp Gln Ile Thr Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu
385  390  395  400

Arg Val Ala Arg Arg Tyr Asp Ala Ala Thr Asp Ser Val Leu Phe Ala
405  410  415

Asn Asn Gln Ala Tyr Thr Arg Asp Asn Tyr Arg Lys Ala Gly Met Ala
420  425  430

Tyr Val Ile Glu Asp Leu Leu His Phe Cys Arg Cys Met Tyr Ser Met
435  440  445

Met Met Asp Asn Val His Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe
450  455  460

Ser Asp Arg Pro Gly Leu Glu Gln Pro Leu Leu Val Glu Asp Ile Gln
465  470  475  480

Arg Tyr Tyr Leu Asn Thr Leu Arg Val Tyr Ile Leu Asn Gin Asn Ser
485  490  495

 Ala Ser Pro Arg Gly Ala Val Ile Phe Gly Glu Ile Leu Gly Ile Leu
500  505  510

Thr Glu Ile Arg Thr Leu Gly Met Gin Asn Ser Asn Met Cys Ile Ser
515  520  525

Leu Lys Leu Lys Arg Lys Leu Pro Pro Phe Leu Glu Glu Ile Trp
530  535  540

Asp Val Ala Asp Val Ala Thr Thr Ala Thr Pro Val Ala Ala Glu Ala
545  550  555  560

Pro Ala Pro Leu Ala Pro Ala Pro Ala Arg Pro Ala Thr Val
565  570  575

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 948 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Spodoptera exigua

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AGGCGCGAGT GCCGTTGGGCC AGAAAACCAAG TGTGCAATGA AAAGGAAAGA GAAAAAGGCA
CAAAAGGAAA AAGACAAAGG GCCAGTCAGT ACAACGCACG TGGATGATCA CATGCCTCCC 120
ATTATGCACT GTGATCCACC GCCCTCCAGG GCCGCAAGAA TTTACCGAGT GGTGCCACGA 180
TTCTCTGAAATG AAAAGCTAAAT GAAGACGAGA AGGCTCAAGA ATGTGCCCATCTCACTGCCAA 240
CCAGAAGTCC TTAATATGCG GGCCTGTCTG GTACCAAGAA GGCATAGAAC AGCCATCAGA 300
AGAGGATCTCA AAAAGAGTCAA CACAGTGCCA GGAAGACGCAA GAAAGTCCGGT ACATGCGCGTT 360
CGGTCAGATC ACCAGAGATGA CGATCCTCAC AGTGCAGCTC ATTGGTGAAT TCGCTAAGGG 420
CCTACCAGCGT TCGCAGAAGA TCCACAGAC GGATCAGATC ACATATTAA AGGCCTGTTTC 480
GAGTAGGAGT ATGATGGTGC GAGTACCTCG GCCGGTGAGA GCACGGGACG AAGCATTGTTT 540
GTTGCGCAAC AACCAGGGCT ACACCCCGCA CAACTACCGC AAGCCAGGCA TGGCCCTACGT 600
CATCGAGGAC CTGCTGCACT TCTGGCGCTGT CATGTACTCC ATGATGATGG AATAACGTCGA 660
CTATGCACTG CTAATTGCCTAG TCGTCATATT CTCAGACGGA CCCGGGCTTG AGCTAACCCT 720
GTTGCTGAGG GACATCCAGA GATATAACCT GAACACGCTG CCGGTGTACA TCTCTGAACCA 780
GAACAGTCGG TCGCCGCTGT GCCCTGTCTAT CTACGGTAAG ATCCTCGGCA TCTTGACGGA 840
GCTGCGACG ACTGCCAGAG AAGAACCAGA CATGTCGATC TCACCTCAAGC TGAAGACACG 900
GAACGTCGCA CCGTTCTTCG AGGATATCGT GGAGCTCCTTC GAGTAAAA 948

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 319 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Arg Pro Glu Cys Val Val Pro Glu Asn Glu Cys Ala Met Lys Arg Lys
1  5  10  15
Glu Lys Lys Ala Gln Arg Glu Lys Asp Lys Leu Pro Val Ser Thr Thr
20 25 30
Thr Val Asp Asp His Met Pro Pro Ile Met Gln Cys Asp Pro Pro Pro
35 40 45
Pro Glu Ala Ala Arg Ile Leu Glu Cys Val Gln His Glu Val Val Pro
50 55 60
Arg Phe Leu Asn Glu Lys Leu Met Glu Gln Asn Arg Leu Lys Asn Val
65 70 75 80
Pro Pro Leu Thr Ala Asn Gln Lys Ser Leu Ile Ala Arg Leu Val Trp
85 90 95
Tyr Gln Glu Gly Tyr Glu Gln Pro Ser Glu Glu Asp Leu Lys Arg Val 100 105 110
5
Thr Gln Ser Asp Glu Asp Glu Asp Ser Asp Met Pro Phe Arg Glu 115 120 125
Ile Thr Glu Met Thr Ile Leu Thr Val Gln Leu Ile Val Glu Phe Ala 130 135 140
10
Lys Gly Leu Pro Gly Phe Ala Lys Ile Ser Gln Ser Asp Gln Ile Thr 145 150 155 160
Leu Leu Lys Ala Cys Ser Ser Glu Val Met Met Leu Arg Val Ala Arg 165 170 175 180
15
Arg Tyr Asp Ala Ala Thr Asp Ser Val Leu Phe Ala Asn Asn Gln Ala 185 190
20
Tyr Thr Arg Asp Asn Tyr Arg Lys Ala Gly Met Ala Tyr Val Ile Glu 195 200 205
Asp Leu Leu His Phe Cys Arg Cys Met Tyr Ser Met Met Met Asp Asn 210 215 220 225
25
Val His Tyr Ala Leu Leu Thr Ala Ile Val Ile Phe Ser Asp Arg Pro 230 235 240
Gly Leu Glu Gln Pro Leu Leu Val Glu Glu Ile Gln Arg Tyr Tyr Leu 245 250 255
30
Asn Thr Leu Arg Val Tyr Ile Leu Asn Gln Asn Ser Ala Ser Pro Arg 260 265 270 275
35
Gly Ala Val Ile Phe Gly Glu Ile Leu Gly Ile Leu Thr Glu Ile Arg 280 285
Thr Leu Gly Met Gln Ser Asn Met Cys Ile Ser Leu Lys Leu Lys 290 295 300 305
40
Lys Arg Lys Leu Pro Pro Phe Leu Glu Glu Ile Asp Trp Asp Val 310 315
CLAIMS

1. DNA comprising the sequence shown in Seq ID No. 2.

2. DNA comprising the sequence shown in Seq ID No. 3.

3. DNA comprising the sequence shown in Seq ID No. 4.

4. DNA comprising a sequence which shows 60% or more homology with the sequence shown in Seq ID No 1, 2 or 3.

5. DNA according to claim 4 wherein said homology is in the range of 65% to 99%.

6. DNA which hybridises to the sequence shown in Seq. ID No. 2, 3 or 4, and which codes for at least part of the Heliothis ecdysone receptor.

7. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 1 to 6 and which codes for a polypeptide which is at least part of the Heliothis ecdysone receptor.

8. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the Heliothis ecdysone receptor ligand binding domain.

9. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the Heliothis ecdysone receptor ligand binding domain.

10. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the Heliothis ecdysone receptor ligand binding domain.

11. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 8, 9 or 10.

12. DNA according to claim 11 wherein said homology is in the range of 65% to 99%.

13. DNA which hybridises to the DNA of any one of claims 8 to 12 and which codes for at least part of the Heliothis ecdysone receptor ligand binding domain.
14. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 8 to 12 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor ligand binding domain.

15. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.

16. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.

17. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.

18. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 15, 16 or 17.

19. DNA according to claim 18 wherein said homology is in the range of 65% to 99%.

20. DNA which hybridises to the DNA of any one of claims 15 to 19 and which codes for at least part of the *Heliothis* ecdysone receptor DNA binding domain.

21. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 15 to 19 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor DNA binding domain.

22. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.

23. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.

24. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.

25. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 22, 23 or 24.
26. DNA according to claim 25 wherein said homology is in the range of 65% to 99%.

27. DNA which hybridises to the DNA of any one of claims 22 to 26 and which codes for at least part of the *Heliothis* ecdysone receptor transactivation domain.

28. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 22 to 26 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor transactivation domain.

29. DNA comprising part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.

30. DNA comprising part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.

31. DNA comprising part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.

32. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 29, 30 or 31.

33. DNA according to claim 32 wherein said homology is in the range of 65% to 99%.

34. DNA which hybridises to the DNA of any one of claims 29 to 33 and which codes for at least part of the *Heliothis* ecdysone receptor hinge domain.

35. DNA which is degenerate as a result of the genetic code of the DNA of any one of claims 29 to 33 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor hinge domain.

36. DNA having part of the sequence shown in Seq ID No. 2, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

37. DNA having part of the sequence shown in Seq ID No. 3, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.
38. DNA having part of the sequence shown in Seq ID No. 4, and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

39. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 36, 37 or 38.

40. DNA according to claim 39 wherein said homology is in the range of 65% to 99%.

41. DNA which hybridises to the DNA of any one of claims 36 to 40 and which codes for at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

42. DNA which is degenerate as a result of the genetic code of the DNA of any one of claims 36 to 40 and which codes for a polypeptide which is at least part of the *Heliothis* ecdysone receptor carboxy terminal region.

43. A polypeptide comprising the *Heliothis* ecdysone receptor or a fragment thereof, wherein said polypeptide is substantially free from other proteins with which it is ordinarily associated, and which is coded for by the DNA of any preceding claim.

44. A polypeptide comprising the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof.

45. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor ligand binding domain.

46. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor DNA binding domain.

47. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor transactivation domain.

48. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor hinge domain.
49. A polypeptide comprising part of the amino acid sequence shown in Seq ID No. 4 or any allelic variant or derivative thereof, which sequence provides the *Heliothis* ecdysone receptor carboxy terminal region.

50. A polypeptide according to any one of claims 44 to 49 wherein said derivative is a homologous variant which includes conservative amino acid changes.

51. DNA comprising the sequence shown in Seq ID No. 6.

52. DNA comprising a sequence which shows 60% or more homology with the sequence shown in Seq ID No. 6.

53. DNA according to claim 52 wherein said homology is in the range of 65% to 99%.

54. DNA which hybridises to the DNA sequence shown in Seq ID No. 6 and which codes for at least part of *Spodoptera* ecdysone receptor.

55. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 51 to 54.

56. DNA comprising part of the sequence shown in Seq ID No. 6, and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.

57. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 56.

58. DNA according to claim 57 wherein said homology is in the range of 65% to 99%.

59. DNA which hybridises to the DNA of any one of claims 56 to 58 and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.

60. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 56 to 58 and which codes for at least part of the *Spodoptera* ecdysone receptor ligand binding domain.
61. DNA comprising part of the sequence shown in Seq ID No. 6, and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.

62. DNA comprising a sequence which shows 60% or more homology with the sequence of claim 61.

63. DNA according to claim 62 wherein said homology is in the range of 65% to 99%.

64. DNA which hybridises to the DNA of any one of claims 61 to 63 and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.

65. DNA which is degenerate as a result of the genetic code to the DNA of any one of claims 61 to 63 and which codes for at least part of the *Spodoptera* ecdysone receptor hinge domain.

66. A polypeptide coded for by the DNA of any one of claims 51 to 65.

67. A fusion polypeptide comprising the polypeptide of claim 45 or 50 (when dependent upon claim 45) and functionally linked to a DNA binding domain and a transactivation domain.

68. Recombinant DNA comprising the DNA of any one of claim 8 to 14 functionally linked to DNA encoding a DNA binding domain and a transactivation domain.

69. A fusion polypeptide according to claim 67 or recombinant DNA according to claim 68 wherein the DNA binding domain and/or transactivation domain is fungal, bacterial, plant or mammalian.

70. A fusion polypeptide or recombinant DNA according to claim 69 wherein the DNA binding domain is GAL4 or A1CR/A.

71. A fusion polypeptide or recombinant DNA according to claim 69 or 70 wherein the transactivation domain is VP16.

72. A fusion polypeptide or recombinant DNA according to claim 69 wherein the DNA binding domain and/or transactivation domain is from a steroid receptor superfamily member.
73. A fusion polypeptide or recombinant DNA according to claim 72 wherein the DNA binding domain and/or transactivation domain is from a glucocorticoid or a *Spodoptera* ecdysone receptor.

74. A recombinant DNA construct comprising recombinant DNA of any one of claims 68 to 73; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.

75. A fusion polypeptide comprising the polypeptide of claim 46 or 50 (when dependent upon claim 46) and functionally linked to a ligand binding domain and a transactivation domain.

76. Recombinant DNA comprising the DNA of any of claims 15 to 21 functionally linked to DNA encoding a ligand binding domain and a transactivation domain.

77. A fusion polypeptide according to claim 75 or recombinant DNA according to claim 76 wherein the ligand binding domain and/or transactivation domain is fungal, bacterial, plant or mammalian.

78. A fusion polypeptide or recombinant DNA according to claim 77 wherein the transactivation domain is VP16.

79. A fusion polypeptide or recombinant DNA according to claim 77 wherein the ligand binding domain and/or transactivation domain is from a steroid receptor superfamily member.

80. A fusion polypeptide or recombinant DNA according to claim 79 wherein the ligand binding domain and/or transactivation domain is from a glucocorticoid or *Spodoptera* ecdysone receptor.

81. A recombinant DNA construct comprising recombinant DNA of any one of claims 76 to 80; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.
82. A fusion polypeptide comprising the polypeptide of claim 47 or 50 (when dependent upon claim 47) and functionally linked to a ligand binding domain and a DNA binding domain.

83. Recombinant DNA comprising the DNA of any one of claims 22 to 28 functionally linked to DNA encoding a ligand binding domain and a DNA binding domain.

84. A fusion polypeptide according to claim 82 or recombinant DNA according to claim 83 wherein the ligand binding domain and/or DNA binding domain is fungal, bacterial, plant or mammalian.

85. A fusion polypeptide or recombinant DNA according to claim 84 wherein the DNA binding domain is GAL4 or A1CR/A.

86. A fusion polypeptide or recombinant DNA according to claim 84 wherein the ligand binding domain and/or DNA binding domain is from a steroid receptor superfamily member.

87. A fusion polypeptide or recombinant DNA according to claim 86 wherein the ligand binding domain and/or DNA binding domain is from a glucocorticoid or Spodoptera ecdysone receptor.

88. A recombinant DNA construct comprising recombinant DNA of any one of claims 82 to 87; and DNA which codes for a gene operably linked to a promoter sequence and a hormone response element, which is responsive to the DNA binding domain coded for by said recombinant DNA.

89. A recombinant DNA construct comprising DNA according to any one of claims 1 to 7; and DNA comprising a sequence which codes for a gene operably linked to a promoter sequence and at least one hormone response element which is responsive to the DNA binding domain coded for by said DNA of any one of claim 1 to 7.

90. A recombinant DNA construct according to any one of claims 74, 81, 88 and 89 wherein said promoter sequence codes for a constitutive, spatially or temporally regulating promoter.
91. A recombinant DNA construct according to any one of claims 74, 81, 88 and 89 wherein there is more than one copy of the hormone response element.

92. A cell transformed with the DNA of any one of claims 1 to 42, and 51 to 65; the polypeptide of any one of claims 43 to 50; the fusion polypeptide of any one of claims 67, 70 to 73, 75, 77 to 80, 82 and 84 to 87; the recombinant nucleic acid of any one of claims 68 to 73, 76 to 80 and 85 to 87; or the recombinant DNA construct of any one of claims 74, 81, 88 and 89.

93. A cell according to claim 92 wherein said cell is a plant, fungal or mammalian cell.

94. A plant, fungus or mammal comprising the recombinant DNA construct of any one of claims 74, 81, 88 and 89.

95. A method of selecting compounds capable of being bound to an insect steroid receptor superfamily member comprising screening compounds for binding to said polypeptide of any one of claims 43 to 50 or the fusion polypeptide of any one of claims 67, 70 to 73, 75, 77 to 80, 82 and 84 to 87, and selecting said compounds exhibiting said binding.

96. A compound selected using the method of claim 95.

97. An agricultural or pharmaceutical composition comprising the compound of claim 96.

98. Use of the compound of claim 96 as an agrochemical or a pharmaceutical.

99. A method of producing a protein, peptide or polypeptide comprising introducing into the cell of claim 92, a compound which binds to the ligand binding domain in said cell.
**Sequence ID 1**

1. TGG CAG GTG AGG AGT TCT TCA GCC GGC GGA GTG TAA CCA CCA AAA ATG
   - ACC TCC CCA CGT TCC TCA AGA AGG CCC CCT ACC ATT GGT TTT TAC

46. CAG TGT ACA TAT GCA AAT TCG GCC ATG CTT GCC AAA TGG ATG TGT
   - GTC ACA TGT ATA CGT TTA AGC CGG TAC GAA CGG TTT ACC TAT ACA

91. ATA TGG GAA AAT GCC AAG AGT A
   - TAT ACG CCT TTA CGG TTC TCA T
811 GAG  GGA  AAA  AGA  CAA  ATT  GCC  CGT  CAG  TAC  GAC  GAC  AGT  AGA  CGA  
    CTC  CCT  TTT  TCT  GTT  TAA  CGG  GCA  GTC  ATG  CTG  CTG  TCA  TCT  GCT  
856 TCA  CAT  GCC  TCC  CAT  CAT  GCA  ATG  TGA  CCC  TCC  GCC  CCC  AGA  GGC  
    AGT  GTA  CGG  AGG  GTA  GTA  CGT  ACT  GGG  AGG  CGG  GGG  TCT  CCG  
901 CCG  TAG  AAT  TCT  GGA  ATG  TGT  GCA  GCA  CGA  CGG  GGT  GGT  GCC  ACG  ATT  
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1126 CCG  TCA  GAT  TAC  CGA  GAT  GAC  GAT  TCT  CAC  AGT  GCA  GCT  CAT  CGT  
    GGC  AGT  CTA  ATG  GCT  CTA  CTG  CTA  AGA  GTG  TCA  CTG  CGA  GTA  GCA  
1171 AGA  ATT  CGC  TAA  GGG  CCT  CCC  GGG  CTC  CGC  CAA  GAT  CTC  GCA  GTC  
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1216 GGA  CCA  GAT  CAC  GTT  ATT  AAA  GCC  GTG  CTC  AAG  TGA  GGT  GAT  GAT  
    CCT  GGT  CTA  GTG  CAA  TAA  TTT  CCG  CAC  GAG  TTC  ACT  CCA  CTA  CTA  
1261 GCT  CCG  AGT  GGC  TCG  GCG  GTA  TGA  CGC  GGC  CAC  CGA  CAG  CGT  ACT  
    CGA  GGC  TCA  CCG  AGC  CGC  CAT  ACT  GCG  CCG  GTG  GCT  GTC  GCA  TGA
| 1306 | GTT | CGA | CAA | CGA | CCA | GCA | GTA | CAC | TCG | CGA | CAA | GTA | CTA | CCT | GCT | TTT | CTT | CTG | TCG | TGG | GCA |
| 1351 | AGG | CAT | CTC | CAT | GAT | TTA | AAT | TAA | GAT | TGA | CTA | AAT | TCT | CTA | CTG | GTA | GAA | GTC | AAT | TAA | CTA |
| 1396 | CAG | GAT | GAG | GAT | TTA | ATG | TTA | GAG | GAT | TGA | GAA | CAC | GCT | GCT | GAG | GAA | GCT | AAT | TAA | CTA |
| 1441 | GTA | TTA | GGT | GTA | AAT | GGA | GGA | GTA | AAT | GGT | GTA | AAT | GGA | GGA | GTA | AAT | GGA | GGA | GTA | AAT | GGA |
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| 1531 | GTT | GTA | CAA | CCA | CTT | CTT | GCT | CTT | CTT | GCT | CTT | CTT | GCT | CTT | CTT | CTT | CTT | CTT | CTT | CTT | CTT |
| 1576 | GCC | GTT | GTC | GGT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT |
| 1621 | CTC | GAT | CTT | CTG | GTA | GTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA | CTA |
| 1666 | GCC | GTT | GTC | GGT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT |
| 1711 | GCC | GTT | GTC | GGT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT | GCT |
Fig. 2 iv

Total number of bases is: 1934.
The sequence shown below is that of pSK16.1

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**Fig. 3.**

**Sequence ID3**

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Fig. 3 vi.

CAC ACA GCA GCT ATC GAA GGT GTG CTC GTT CGG AAA ACA AAT TCA
2431 GAT TTA CTG ACA TGG ACA CTC GAC CCG GAA CTT C
CTA AAT GAC TGT ACC TGT GAG CTG GCC CTT GAA G

Total number of bases is: 2464.

Fig. 4.

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Fig. 4 iii.

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790 800 810 820 830 840
TCTTCAGGCCGGAGTGAACCAAAATGCAGTGTACATATGCAAATTCGGCCATGCTTGGC
F R R S V T K N A V Y I C K F G H A C

850 860 870 880 890 900
AAATGGGATATCTATATGCAGGAGGAAATGTCAGGAGTGTCGTTGAAGAAATGTCTTTGGC
E M D I Y M R K C Q E C R L K K C L A

910 920 930 940 950 960
TGGGCATGAGGCAGGGCTTGGGCTGCTCAGTGAACGAGTGTCGATGAACCGGAAAGAGA
V G M R P E C V V P E N Q C A M K R K E

970 980 990 1000 1010 1020
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K K A Q R E K D K L P V S T T T V D D H
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Fig. 4 v.

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QLIVEFAKGLPGFAKISQSD

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** Fig. 5 ii. **

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PMGNVGVGVGVGGNVSMYANAQTMALMGVALHSHQQQLIGGVAVKSEH
**Fig. 6.**

Chemical

| Transactivation Domain | DNA Binding Domain ++ | EcR* |

| Hormone Response Element | M** | Reporter Gene (GUS) |

++ Glucocorticoid receptor DNA binding and transactivation domains

* Insect ecdysone ligand binding domain

** Minimal 35S CaMV promoter
Fig. 10.

pMF6HG1PAT
8776 bps

EcoRI

CaMV 35S
ori

Aah Intron I
8000

XhoI...

HG1

6000

NOS

AdhIntron I
4000

35SCaMV I

NOS

amp

2000

BAR

W0 96/37609

PCT/GB96/01195

SUBSTITUTE SHEET (RULE 26)
Fig. 11.

pMF6GREcRS
7176 bps

Ori

7000 CaMV 3S

GR

Adh Intron 1

Sall

6000

Eagl SfiI NotI

PstI Sall

5000 SacI

4000 KpnI SacII

3000 ClaI EcoRI

2000 PstI

1000 XhoI

amp

BglII

NOS 3'

7000 CaMV 35S

BamHI

SUBSTITUTE SHEET (RULE 26)
Fig. 22.
Fig. 24.

42/56

GUS Units

0 100 200 300 400 500

Reporter+ DMSO
Reporter+ Effector+ DMSO
Reporter+ Effector+ 10μM Mimic
No DNA

Fig. 26.

Response Element for GR

B-globin Promoter
Hind III
Lac Z
Eco RI
Spe I
Aat II
Afl III
Sal I

pSW GRE4

Amp
NEO
TK
BamHI
Asp 718

SUBSTITUTE SHEET (RULE 26)
Fig. 25.

pcDNA3GRHecR 8100 bps

8098, KpnI, BamHI, 1
8082, HindIII, EcoRI, 6
Sall, 403
CVM Promoter
GR
Amp
Col EI
SV40 poly A
Neomycin
PSV40 ori
BGH poly A
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KpnI, 1818
EcoRI, 1949
Xhol, 2459
BamHI, 2700
EcoRI, 2731
Xhol, 2764
XbaI, 2776
Fig. 36.

pcDNA3GRVP16HEcR
7600 bps

BamHI, 1
EcoRI, 6
Sall, 403

CVM Promoter
GR1
VP16
GR2

Amp
Col EI
SV40 poly A
Neomycin
PSV40 ori

BGH poly A
fl ori

XhoI, 2459
XhoI, 2764
XbaI, 2776

BamHI, 2700
EcoRI, 2731

KpnI, 1818
EcoRI, 1949

EcoRI, 1675
Fig. 37.
Fig. 38.
Spodoptera exigua DNA sequence.

Fig. 40.

Spodoptera exigua Hinge and Ligand Binding Domains

Sequence ID 6

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Fig. 40 ii.

766 GTA CAT CCT GAA CCA GAA CAG TCG GTC GCC GTG CTG CCC TGT CAT CAT GTA GGA CTT GGT CTT GTC AGC CAG CGG CAC GAC GGG ACA GTA

811 CTA CGC TAA GAT CCT CGG CAT CCT GAC GGA GCT GCG GAC CCT GGG GAT GCG ATT CTA GGA GCC GTA GGA CTG CCT CGA CGC CTG GGA CCC

856 CAT GCA GAA CTC CAA CAT GTG CAT CTC ACT CAA GCT GAA GAA CAG GTA CGT CTT GAG GTT GTA CAC GTA GAG TGA GTT CGA CTT CTT GTC

901 GAA CGT GCC GCC GTT CTT CGA GGA TAT CTG GGA GTG CCT CGA GTA CTT GCA CGG CGG CAA GAA GCT CCT ATA GAC CCT GCA GGA GCT CAT

946 AAA TTT

Total number of bases is: 948.
### Fig. 41.

Sequence I.D. 7

**Sequence comparison between Heliothis 19R clone and SECr Taq clone**

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Fig. 42.

![Graph showing GUS Units for different treatments.]

- Reporter+ DMSO
- Reporter+ Effector+ DMSO
- Reporter+ Effector+ 10µM Mimic
- Reporter+ DMSO
- Reporter+ Effector+ DMSO
- Reporter+ Effector+ 10µM Mimic
- No DNA

*p35PAC yielded 2600 GUS Units*
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/85 C12N15/62 C07K14/72 C07K19/00
C12N5/10 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO,A,93 03162 (GENENTECH INC) 18 February 1993</td>
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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Date of the actual completion of the international search

9 August 1996

Authorised officer

Gurdjian, D.
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| X        | CELL, OCT 4 1991, 67 (1) P59-77, UNITED STATES, XP002010069 <br> KOELLE MR ET AL: "The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily."
see the whole document | 4,5, 1-3, 8-43, 45-49, 51-92 |
| Y        | INSECT BIOCHEM MOL BIOL, JAN 1993, 23 (1) P115-24, ENGLAND, XP002010070 <br> IMHOF MO ET AL: "Cloning of a Chironomus tentans cDNA encoding a protein (cEcRH) homologous to the Drosophila melanogaster ecdysteroid receptor (dEcR)."
see the whole document | --- |
see the whole document | 4,5, 52, 53 |
| Y        | EP, A, 0 615 976 (AMERICAN CYANAMID CO) 21 September 1994 <br> see page 6, line 28 - line 32; claims 1-12; example 2 | 8-43, 45-49, 51-92 |
| Y        | EUR. J. ENTOMOL. (1995), 92(1), 333-40 <br> CODEN: EJEN2; ISSN: 1210-5759, XP002010346 <br> SMAGHE, GUY ET AL: "Biological activity and receptor-binding of ecdysteroids and the ecdysteroid agonists RH-5849 and RH-5992 in imaginal wing discs of Spodoptera exigua (Lepidoptera: Noctuidae)"
see page 336, paragraph 3 - page 337, paragraph 2 | 51-65 |
| A        | DEVELOPMENTAL GENETICS, 1995, 17, 319-330, XP002010345 <br> KOTHAPALLI R ET AL: "CLONING AND DEVELOPMENTAL EXPRESSION OF THE ECDSyne RECEPTOR GENE FROM THE SPRUCE BUDWORM, CHORISTONEURA-FUMIFERANA"
see the whole document | 1-5, 51-54 |
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**INTERNATIONAL SEARCH REPORT**

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ✗ Claims Nos.: 98
   - because they relate to subject matter not required to be searched by this Authority, namely:
     - Although this claim is directed partly to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition

2. ☐ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)
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