Selective photochemical treatment of oestrogen receptor in a *Xenopus* liver extract destroys hormone binding and transcriptional activation but not DNA binding

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Photochemical excitation of a simple derivative of oestradiol using light in the UV-A range totally, permanently and selectively inactivates the oestrogen receptor protein present in a *Xenopus* liver extract without affecting its overall size. Inactivation of the binding site proceeds to completion with simple, first-order kinetics. Inactivation is prevented by excess oestradiol but not by non-oestrogenic steroids. Using an *in vitro* transcription system, we show that the treatment eliminates transcription of vitellogenin genes, which are normally oestrogen-responsive, but has no effect on the transcription of albumin genes, which are not. Native receptor binds to the two imperfectly palindromic sequences in the vitellogenin B2 gene which together constitute an oestrogen-response unit. Its affinity for one sequence is greater than its affinity for the other, suggesting that a compulsory binding order operates when receptor interacts with the B2 gene. Photo-inactivated receptor still binds to both sequences, but with reduced affinity. We also discuss our findings in the context of the current concern over the effects of UV-A on human tissues.

Key words: *in vitro* transcription/photosensitive steroid/steroid receptor function/UV-A and photosensitivity

Introduction

Oestrogen antagonists are widely used both clinically in the treatment of oestrogen-dependent human breast cancer and in studies at the molecular level of how oestrogen and its receptor protein activates genes. Those currently available, such as Tamoxifen, suffer from certain disadvantages. Not all oestrogen-dependent tumours respond to Tamoxifen, and it does not always act as a pure antagonist, which limits its value for molecular studies (Westley *et al.*, 1984).

A simple method of selectively and permanently inactivating the oestrogen receptor would be valuable in studies of how the oestrogen receptor functions and might have clinical potential. We describe here a strategy based on attacking the receptor with a photo-sensitive ligand. During attempts to attach ligands covalently to the binding site of rat oestrogen receptor, Katzenellenbogen *et al.* (1974) noticed that UV irradiation in the presence of 6-oxo-

oestradiol inactivates hormone binding. Here, using as a test system the oestrogen-induced activation of vitellogenin genes in *Xenopus* liver (reviewed by Wahli, 1988), we exploit this observation. We show that 6-oxo-oestradiol activates vitellogenin genes but that when excited it attacks both *Xenopus* and human oestrogen receptors selectively, destroying both hormone binding and transcription activation without affecting the overall size. Finally, we examine interactions between receptor and the oestrogen-response unit (ERU) in the vitellogenin B2 gene and the effect of inactivation on this binding.

Results

6-Oxo-oestradiol activates vitellogenin genes

Using competition assays, we found that the affinities of oestrogen receptors for 6-oxo-oestradiol are lower than they are for oestradiol. The dissociation constant of the *Xenopus* receptor is 4.3 nM for 6-oxo-oestradiol, compared to 0.5 nM for oestradiol (Figure 1A). The corresponding figures for the human receptor HE0 are 12.0 and 1.0 nM. In liver cultures, 5 nM oestradiol and 43 nM 6-oxo-oestradiol, which both give 90% saturation of receptor, induce similar levels of vitellogenin mRNA (Figure 1B). 6-Oxo-oestradiol is not metabolized to oestradiol (Figure 1C), and so the induction appears to be a direct effect of the complex which it forms with receptor.

Inactivation of receptor

We filter out light below 295 nm so that we can excite 6-oxo-oestradiol but avoid any possible damage from absorption of light by proteins (Figure 2A). We irradiated liver extracts using just enough 6-oxo-oestradiol to saturate the receptor, and found that this treatment inactivates the binding site with simple, first-order kinetics. In Figure 2B the inactivation reaches 80%, but with longer irradiation times it goes to completion. Excess oestradiol completely prevented inactivation, and the protection found using two intermediate concentrations agreed well with that predicted from the affinities of the two ligands. High concentrations (1 μ M) of non-oestrogenic steroids (dexamethasone, progesterone, testosterone), which neither bind to receptor (Westley and Knowland, 1978) nor activate vitellogenin genes (Wangh and Knowland, 1975), had no protective effect (data not shown). We conclude that 6-oxo-oestradiol competes with oestradiol for receptor, and that on excitation it modifies the binding site in such a way that it can no longer bind oestradiol. Similar results were found (Figure 2C) with human receptor. In order to see whether inactivation of the binding site is accompanied by covalent attachment of the ligand, we irradiated extracts containing receptor in the presence of tritiated 6-oxo-oestradiol and assayed for attachment using sucrose gradient centrifugation, gel filtration and gel electrophoresis. We did not find unequivocal evidence for attach-

Fig. 1. 6-Oxo-oestradiol activates vitellogenin genes by binding to receptor. (A) Determination of the affinity of *Xenopus* oestrogen receptor for 6-oxo-oestradiol. Liver extracts containing receptor (Westley and Knowland, 1978) and a fixed concentration (5 nM) of [³H]oestradiol were incubated with unlabelled 6-oxo-oestradiol (●—●) or oestradiol (○—○). After correcting for low-affinity binding at 10⁻⁶ M competitor, the dissociation constant is calculated from the ratios of the concentrations at the inflection points X and Y (Korenman, 1974; Rodbard, 1973). (B) 6-Oxo-oestradiol activates vitellogenin genes in liver cultures. RNA from male liver cultures (Wang and Knowland, 1975) was analysed using a Northern blot and a nick-translated vitellogenin probe. Lane 1, no hormone; lane 2, 5 nM oestradiol; lane 3, 5 nM 6-oxo-oestradiol; lane 4, 43 nM 6-oxo-oestradiol. Full-length vitellogenin mRNA is 6.3 kb long. (C) Metabolism of 6-oxo-oestradiol in liver cultures. [³H]-6-oxo-oestradiol was added at 20 nM to a 10 ml culture containing 1 g of liver. Steroids were extracted from 200 mg samples of tissue and analysed on Sephadex LH-20 (Wright *et al.*, 1983). 6-Oxo-oestradiol was metabolized to unidentified products but not to oestradiol (elution position arrowed). The elution profiles after 2 h (—) and 24 h (---) of incubation are shown.

ment of the ligand to receptor, and the mechanism of inactivation remains uncertain. However, the size of the receptor is not affected (Figure 2D), showing that the treatment does not cleave peptide bonds.

In vitro transcription of vitellogenin sequences and inactivation of transcription

To test for effects on transcription, we used an *in vitro* system from liver nuclei and a vitellogenin B2 plasmid, which starts at -770, contains oestrogen-response elements (EREs) at -300 and -333, and continues to +2 kb. In agreement with Corthésy *et al.* (1988), who found correct, oestradiol-dependent initiation in a similar system, we found that the efficiency of transcription depends critically on the relative concentrations of DNA and protein (data not shown). To test the accuracy of our system we used ribonuclease mapping. Figure 3 shows that female liver generates a protected fragment 53 nt long, representing the first exon. This product is not found in male liver, where the vitel-

logenin genes are inactive, nor in the nuclear extract, but is found after *in vitro* transcription. A 220 nt product (arrowed), representing the initial transcript, is detectable in female liver RNA but was not found using this particular extract. However, in other experiments (e.g. Figure 4), the initial transcript was much more abundant, suggesting that rates of processing are, for unknown reasons, rather variable. The results show that our system initiates transcription accurately and cleaves the initial transcript correctly at the first exon-intron junction.

Irradiation of the extract with 6-oxo-oestradiol completely abolishes oestrogen-dependent transcription, but irradiation alone, or addition of 6-oxo-oestradiol without irradiation, do not (Figure 3, lanes 6–9). Oestradiol present at high concentrations during irradiation prevents the inactivation of transcription, but progesterone does not (Figure 4). These results show that inactivation of the binding site also destroys the ability of the receptor to activate transcription.

Inactivation is selective

We performed two experiments to test the specificity of the treatment. First, we eliminated active receptor from a *Xenopus* liver extract by photochemical treatment (Figure 2B) and then added human receptor made *in vitro*. This restored accurate transcription of the vitellogenin template (Figure 5), confirming that loss of transcription is due to inactivation of receptor, and showing that any other factors required for vitellogenin transcription are not affected. Figure 5 also shows that the treatment inactivates both *Xenopus* and human receptors and that inactivated receptor does not interfere with normal transcription. Next, we tested the transcription of albumin genes, which are expressed in liver in the absence of oestrogen (Kazmaier *et al.*, 1985), using an incubation containing both vitellogenin and albumin templates. Figure 6 shows that increasing treatment eventually abolishes vitellogenin transcription, but that accurate transcription of albumin genes persists. These tests show that the treatment is specific for receptor in a crude extract and strongly suggests that, at the concentrations used here, excited 6-oxo-oestradiol can react only with receptor, and hence that reaction requires close contact between 6-oxo-oestradiol and its target.

Inactivated receptor still binds to target DNA

To see whether inactivated receptor can bind to DNA we used a combination of gel retardation and DNase I footprinting. The B2 gene contains two different, imperfectly palindromic EREs which appear to contribute to an ERU (Klein-Hitpass *et al.*, 1988) and to bind receptor (ten Heggeler-Bordier *et al.*, 1987) but it is not clear whether the interactions are confined to those elements, and their limits have not been mapped in detail. We therefore tested the ability of B2 fragments to displace [³H]-oestradiol-receptor complex from DNA-cellulose. Figure 7 shows that only two fragments, both containing the two elements, did so to any significant extent. We chose the longer one (no. 5), which displayed marginally greater competition, for further analysis, and it formed two complexes with receptor-enriched extracts.

In Figure 8, we explored the interactions between receptor and both strands of this fragment, using both native and photo-inactivated receptor, by indirect DNase I footprinting. Complexes formed with end-labelled fragments were treated

Fig. 2. Inactivation of hormone binding. (A) Absorption and irradiation of 6-oxo-oestradiol. Receptor preparations were irradiated using a Schott WG 320 filter to cut out light below 295 nm (hatched area). The vertical bars represent the relative intensities of the major emission lines of the lamp. E₂, oestradiol; 6-oxo-E₂, 6-oxo-oestradiol. (B) Loss of binding activity during irradiation of *Xenopus* oestrogen receptor. Receptor preparations were irradiated using 43 nM 6-oxo-oestradiol and increasing concentrations of oestradiol, and the surviving binding activity (●—●) was measured at increasing times. A saturating concentration of oestradiol (1 μM) gives 100% protection against inactivation (○—○), and the protection found at 2.7 nM (+) and 11.7 nM (Δ) agrees with the 35 and 70% protection predicted (dotted lines). (C) Inactivation of human receptor. Human receptor synthesized *in vitro* was irradiated using 120 nM 6-oxo-oestradiol either alone (●—●) or with 1 μM oestradiol (○—○). The rates of inactivation of human and *Xenopus* receptors were very similar. (D) SDS gel of receptor after photochemical treatment. Labelled human receptor synthesized *in vitro* was irradiated for 0, 10, 20, 30, 40 and 60 min using 120 nM 6-oxo-oestradiol either alone (lanes 1–6); or with 1 μM oestradiol (same times, lanes 7–12); or was incubated with 120 nM 6-oxo-oestradiol for 0, 30 and 60 min with no irradiation (lanes 13–15). The band at 66 kD is due to full-length receptor. Small amounts of shorter products, probably due to premature termination of translation, are also found.

with DNase I, isolated by gel retardation and analysed on sequencing gels. This revealed clear footprints on the top strand in two overlapping places (lanes 2 and 3), and complementary ones on the bottom strand (lanes 9 and 10), although the bottom-strand footprints were not as clear in the lower portion of the protected sequence. In the complex C1, the protected sequence is TAAAGTCACCTTTGACCAAC, which includes the left-hand ERE (underlined) between -335 and -323. In the complex C2, the protected sequence is TAAAGTCACCTTTGACCAACCAAGTTATCATGACCTCTT, which includes both EREs (underlined). The appearance of the footprints indicates that the complexes are shielded from attack by DNase I over their entire length, and hence that interactions between receptor and the B2 ERU extend somewhat beyond the limits of the EREs identified by functional assays. Martinez and Wahli (1989) found similar results with the B1 ERU. However, we found no evidence for any interactions in more remote regions.

When fragments were incubated with extracts containing photo-inactivated receptor and treated with DNase I, gel-retarded complexes were still found. Photo-inactivation did not reduce the intensities of the signals, and this alone suggests that photo-inactivated receptor still binds to DNA. It is extremely unlikely that the gel-retarded complexes are

due to a small amount of receptor which escapes photo-inactivation because the treatment used eliminates at least 90% of the hormone-binding and transcriptional activity, which would not leave enough normal receptor to form a retarded complex (see Materials and methods). However, gel-retardation assays cannot reveal minor changes in either the affinity of receptor for its target or the pattern of the interaction. We therefore analysed the retarded complexes on sequencing gels. These confirm that the total amount of labelled DNA that interacts with a fixed amount of receptor and is recovered on the sequencing gels is not significantly reduced by photo-inactivation, as is clear from the overall intensities of tracks 15–21 and 22–28. They also show (lanes 15–28) that when excess oestradiol was used to protect receptor from attack by excited 6-oxo-oestradiol, normal footprints were found, as expected (compare lanes 16 and 17 with 18 and 19 for the top strand, and lanes 23 and 24 with 25 and 26 for the bottom strand). With unprotected, and therefore inactivated receptor, the footprints were still detectable but somewhat weaker (lanes 20, 21 and 27, 28), and no single interaction appeared to be particularly affected. The simplest interpretation of these results is that inactivated receptor can still bind to both components of the ERU but that its affinity is slightly reduced. This could explain why normal receptor can restore transcription to an

Fig. 3. Ribonuclease mapping of products of *in vitro* transcription of the vitellogenin B2 gene and the effect of inactivating receptor. (A) The 2.7 kb *Eco*RI fragment of the B2 gene spanning the initiation site (Germond *et al.*, 1983) subcloned into pBR322 and linearized at the *Sph*I site in the vector was transcribed *in vitro*. The products were analysed using the probe indicated. **Lane 1**, undigested probe. **Lanes 2–5**: protected fragments found using female liver (**lane 2**), male liver (**lane 3**), nuclear extract alone (**lane 4**), probe alone (**lane 5**). **Lanes 6–9**: protected fragments found using extracts which had received no treatment (**lane 6**), irradiation and 6-oxo-oestradiol (**lane 7**), irradiation but no 6-oxo-oestradiol (**lane 8**), 6-oxo-oestradiol but no irradiation (**lane 9**). **Lane 10**, blank; **lane 11**, size markers. The arrows mark the protected fragments representing the initial transcript (220 nt) and the first exon (53 nt).

extract containing inactivated receptor (Figure 5); the normal receptor, with higher affinity for the ERU, simply displaces the damaged receptor.

Discussion

This work shows that when photochemical treatment is used to inactivate the hormone-binding site of oestrogen receptor proteins the reaction proceeds to completion with simple, first-order kinetics and destroys the ability of the receptor to activate a target gene. Our conclusions are not affected by the recent finding that the human receptor plasmid HE0 (used here to generate protein) contains a point mutation (Tora *et al.*, 1989), because it is clear that the treatment inactivates both wild-type *Xenopus* receptor and mutant human receptor. In a crude extract, inactivation is specific for receptor, and this could provide a convenient system for testing the transcriptional activity of receptors extracted from other tissues, such as primary human breast tumours.

We do not yet know what the treatment does to the receptor. Like Katzenellenbogen *et al.* (1976), we have not found clear evidence for covalent attachment of 6-oxo-

Fig. 4. Protection of the receptor binding site in relation to inactivation of transcription. Nuclear extracts were irradiated and the products of transcription were characterized by ribonuclease mapping. **Lanes 1–3**: protected fragments found using RNA from female liver (**lane 1**), untreated extract without template (**lane 2**), or with template (**lane 3**). **Lanes 4–6**: protected fragments found using extract which had been irradiated using 43 nM 6-oxo-oestradiol alone (**lane 4**), 43 nM 6-oxo-oestradiol plus 1 μ M oestradiol (**lane 5**), or 43 nM 6-oxo-oestradiol plus 1 μ M progesterone (**lane 6**). **Lane 7**, markers. The arrows at 220 and 53 nt mark the positions of protected fragments representing the initial transcript and the first exon.

Fig. 5. Human receptor can replace *Xenopus* receptor and is itself susceptible to inactivation. Human receptor synthesized *in vitro* was added to nuclear extracts containing inactivated receptor before *in vitro* transcription and ribonuclease mapping. **Lane 1**, undigested probe; **lane 2**, digested probe. **Lanes 3 and 4**: protected fragments found using untreated nuclear extract without template (**lane 3**), or with template (**lane 4**). There is slight spill-over from lane 4 to lane 3. **Lanes 5–7**: extract which had been irradiated using 43 nM 6-oxo-oestradiol was used for transcription with no additions (**lane 5**), with an amount of human receptor equivalent to that inactivated (**lane 6**), or with the addition of human receptor followed by a further irradiation with 6-oxo-oestradiol (**lane 7**). The arrows mark the positions of protected fragments representing the initial transcript (220 nt) and the first exon (53 nt).

oestradiol, and the question remains open. Inactivated receptor is the same size as native receptor, showing that the treatment does not cleave peptide bonds, and it can still recognize its DNA target, even though it binds less strongly, suggesting that the overall damage is not very extensive. Recent work (Webster *et al.*, 1989) suggests that the activation function may not lie within a single, discrete domain, but that it is created by precise folding of dispersed elements. If so, identification of the residues affected, which has been achieved in other systems (Martyr and Benisek,

Fig. 6. Inactivation of receptor in liver extracts eliminates vitellogenin transcription but does not affect albumin transcription. The effects of inactivation were studied by ribonuclease mapping using the vitellogenin template shown in Figure 3 and an albumin template derived from the 68 kd gene (May *et al.*, 1982). This template runs from -49 (*Bgl*II) to +607 (*Acc*I) and covers the first exon (117 nt), the first intron, and 4 nt of the second exon. **Lane 1**, extract alone, both probes used. **Lane 2**, vitellogenin template and vitellogenin probe. The band at 220 nt represents the unspliced transcript; in this extract the amount of spliced product (53 nt) was very small and it is not shown. **Lanes 3-6**: both templates (0.5 μ g of each) and both probes using extracts (12.5 μ l containing 44 μ g of total protein) which had received no treatment (**lane 3**), or enough to inactivate 30% (**lane 4**), 70% (**lane 5**) or 99% (**lane 6**) of the receptor. **Lane 7**, albumin template and albumin probe; no treatment. The signals here are stronger than those in lanes 3-6 because when a single template is used it is necessary to add more to maintain the correct ratio of DNA to extract. The bands at 607 and 117 nt represent the unspliced and spliced albumin transcripts.

1975; Ogez *et al.*, 1977), would be valuable for identifying the essential amino acid(s) in the activation function. Our current understanding of this function is based on making truncated receptors, and the evidence is somewhat contradictory. For example, Green *et al.* (1987) suggest that the presence of an intact hormone-binding domain is essential for efficient stimulation of gene transcription, while Waterman *et al.* (1988) find that it is not.

Our work is also relevant to the question of how receptor binds to EREs in vitellogenin genes. Two groups, both using methylation interference, have studied the single, almost perfectly palindromic ERE in the vitellogenin A2 gene and have established that this element (GGTCACAGTGACC) binds receptor (Metzger *et al.*, 1988; Klein-Hitpass *et al.*, 1989), probably as a dimer. When two such EREs were placed upstream of a tk promoter, they acted synergistically to activate a CAT reporter gene (Klein-Hitpass *et al.*, 1988) and this gave rise to suggestions that the elements bind receptor co-operatively (Tsai *et al.*, 1989). Constructs containing two copies of the almost perfectly palindromic A2 sequence do indeed seem to bind receptor co-operatively (Klein-Hitpass *et al.*, 1988), while others containing sequences similar, but not identical, to the elements present in the B1 and B2 genes do not (Klein-Hitpass *et al.*, 1988). However, none of the combinations tested in those experiments actually exist in any vitellogenin gene. In view of the demonstration by the same authors that single base changes can greatly reduce the activity of an ERE (Klein-Hitpass *et al.*, 1988), it is essential to use the naturally existing sequences in any test of the binding mechanism. In detailed studies, Martinez and Wahli (1989) have used the B1 gene, which contains two closely linked EREs and a third much further upstream (Walker *et al.*, 1984), concentrating on the linked elements. We have studied the B2 gene, which contains just two, closely linked EREs.

In a simple co-operative binding model, the initial affinities of the two unoccupied regions are identical, so that receptor can bind to either. Then, following the first interaction, the affinity of the unoccupied region increases. Such a model predicts the formation of three complexes. One, due to occupation of both regions, would have the lowest electrophoretic mobility and both regions would be protected against

Fig. 7. Complexes formed between receptor-enriched extracts of liver nuclei and vitellogenin B2 sequences. The left panel shows the fragments used to test for interaction with receptor and the middle one their ability to displace the oestradiol-receptor complex from DNA-cellulose. Significant displacement is seen with fragments 5 (■) and 6 (▲), but not with fragments 1 (○), 2 (△), 3 (●) and 4 (□). The right panel shows gel-retarded complexes formed using receptor-enriched extract and fragment 5. **Lane 1**, end-labelled fragment alone. **Lanes 2-4**: complexes found using extract, 0.2 ng of fragment, and poly(dI-dC):poly(dI-dC) at 10 ng (**lane 2**), 100 ng (**lane 3**) or 1 μ g (**lane 4**).

Fig. 8. DNase I footprints detected on the B2 gene using native and photo-inactivated receptor. **Lanes 1–14:** untreated receptor. Complexes formed using receptor-enriched extracts of liver nuclei and fragment 5 from Figure 7 were treated with DNase I, separated from free DNA by gel retardation, and run on sequencing gels. **Lanes 1–7:** top strand; DNase I products found with free DNA (lane 1), the complexes C1 (lane 2) and C2 (lane 3), and the Maxam–Gilbert products after cleavage at: C + T (lane 4); C (lane 5); G + A (lane 6); G (lane 7). **Lanes 8–14:** bottom strand; DNase I products found with free DNA (lane 8), the complexes C1 (lane 9) and C2 (lane 10), and the Maxam–Gilbert products (lanes 11–14; chemistry as in lanes 4–7). **Lanes 15–28:** effect of photo-inactivation of receptor. Complexes formed between the DNA fragment and photo-inactivated receptor were treated with DNase I, separated from free DNA by gel retardation, and the DNA in them was applied to sequencing gels. **Lanes 15–21:** top strand, DNase I products found with free DNA (lane 15), the complexes C1 (lane 16) and C2 (lane 17) formed with native receptor; the complexes C1 (lane 18) and C2 (lane 19) formed with receptor preparations which had been irradiated using 6-oxo-oestradiol (43 nM) and protecting oestradiol (1 μ M); and the two corresponding complexes C1 (lane 20) and C2 (lane 21) formed with receptor which had been irradiated using 6-oxo-oestradiol (43 nM) alone. **Lanes 22–28:** the complexes and treatments correspond to those given for lanes 15–21 but the lower strand was labelled.

DNase I. Since any binding model can account for the formation of this complex (C2 in our experiments) one cannot test for co-operative binding by studying it. However, two smaller complexes, each due to interactions between receptor and one single region, would also be formed. They would co-migrate in gel-retardation experiments, and form a mixed complex (C1). In such a mixture, both regions would be protected against DNase I, but Figure 8 shows that this is not found in practice, and that the DNA in the complex C1 is protected in only one region. We conclude that receptor interacts with either the left-hand region of the B2 ERU alone, or with both regions; but not with the right-hand region alone. This shows that the unoccupied left-hand element has a significantly higher affinity for receptor, resulting in a compulsory binding order for the initial interaction, with the left-hand element being occupied first. Martinez and Wahli (1989) found exactly similar results with the B1 gene. We conclude that a simple co-operative binding model, in which unoccupied elements have identical affinities for receptor, does not apply to either the B1 or the B2 gene. However, Martinez and Wahli also show that the affinity of the receptor for the right-hand element increases after the left-hand one has been occupied. Thus, the initial interaction between receptor and the ERU takes place by a compulsory-order binding mechanism, but the subsequent increase in the amount of receptor bound to the ERU includes a co-operative mechanism.

Finally, this work may be relevant to current concern over the effects of UV-A light on human tissues. The light intensities we use are relatively low. Between 300 and

400 nm the overall intensity is $\sim 90 \text{ W/m}^2$, with 80% of that falling in the UV-A range (315–400 nm). That is not grossly different from the figure of 10–40 W/m^2 found between 290 and 400 nm in direct sunlight at sea level at moderate latitudes (Gast, 1960). It is also noticeable that the wavelengths and amounts of light ($3 \times 10^5 \text{ J/m}^2$) that we use to inactivate the receptor are similar to those used by Tyrrell and Pidoux (1989) to inactivate human fibroblasts in culture. A certain amount of UV-A does penetrate human skin (Everett *et al.*, 1966), and a recent review (Roza *et al.*, 1989) points out that transmission of UV-A through the epidermis into the dermis is a significant factor in induction of genetic damage, skin cancer and photoaging. Roza *et al.* also suggest that a variety of naturally occurring molecules, including steroids, could perhaps act as mediators of the effects of UV-A. We show here that one steroid at least can be excited by UV-A and attack an important cellular factor. Although we do not know whether such photochemical processes do occur in human tissue, it would be interesting to examine the effects of light on human cells which are exposed to steroids.

Materials and methods

Oestrogen receptors

Liver extracts containing receptor were prepared and assayed as previously described (Westley and Knowland, 1978; Wright *et al.*, 1983). Human receptor was prepared by *in vitro* translation (Kumar *et al.*, 1986) and precipitation with 60% ammonium sulphate.

Irradiation of extracts

The irradiation apparatus consists of a 1 cm quartz cuvette enclosed in a jacket through which cooling water (8°C) circulates and which bears a filter mounted in front of a medium-pressure mercury vapour lamp. The bulb is 10 cm from the filter and the filter is 12 mm from the cuvette. To estimate the intensity of light which excites 6-oxo-oestradiol (between ~300 and ~400 nm) we used two irradiations with (i) a 2 mm Schott WG320 filter, cutting off at 295 nm, and (ii) a Kodak 2E gelatin filter, cutting off at 415 nm. From chemical actinometry (Hatchard and Parker, 1956) with 6 mM potassium ferrioxalate in the cuvette and the relative intensities of the main emission lines of the lamp we estimate the intensity to be 1.57×10^{16} photons/s/cm².

Preparation of tritiated 6-oxo-oestradiol

[2,4,7,16,17-³H]6-oxo-oestradiol (115 Ci/mmol) was custom-synthesized by Amersham essentially as described (Longwell and Wintersteiner, 1940; Knuppen and Hoppen, 1982). [2,4,7,16,17-³H]oestradiol diacetate was oxidized to the 6-oxo derivative with chromium trioxide. After alkaline hydrolysis to remove the acetate groups the product was purified by preparative TLC.

In vitro transcription

An *in vitro* transcription system was prepared from female liver nuclei by gently stirring them in extraction buffer followed by passage over Sephadex LH-20 (to remove endogenous oestrogens) and precipitation with 1.5 vol of saturated ammonium sulphate. The precipitate from 1 g of liver was dissolved in 200 µl of low-salt extraction buffer (Wright *et al.*, 1983). Samples were irradiated as required, and transcriptions (final volume 20 µl, 2.2 mg/ml of protein) contained 12.5 µl of extract (1 fmol of receptor), 1 µg of template, 50 nM oestradiol, 33 mM HEPES, pH 7.9, 60 mM KCl, 10% glycerol, 6 mM MgCl₂, 5 mM creatine phosphate, 0.6 mM NTPs, 0.6 mM DTT, 0.06 mM Na₂EDTA and lasted for 3 h at 30°C.

Probes

A uniformly labelled anti-sense vitellogenin B2 probe extending from -42 to +220 (Figure 3) was synthesized using an SP6 transcription system. An anti-sense albumin probe was prepared using T7 RNA polymerase and a -49 to +1100 nt (*Bam*HI) fragment of the 68 kd gene (May *et al.*, 1982) subcloned into Bluescribe (Vector Cloning Systems), linearized at the *Hind*III site downstream of the *Bgl*II site.

Hybridization and ribonuclease treatment

Probes were hybridized overnight in 31 µl of 80% formamide, 0.4 M NaCl, 40 mM PIPES, 1 mM Na₂-EDTA at 40°C to RNA from male and female liver or to the products of transcription. Three hundred microlitres of 10 mM Tris-HCl, pH 7.5, 5 mM Na₂EDTA, 300 mM NaCl containing 40 µg/ml RNase A and 2 µg/ml RNase T₁ were added and samples were digested for 1 h at 35°C followed by digestion with 20 µl of 10% SDS and 50 µg of proteinase K and phenol extraction. Samples were run on 8% sequencing gels.

DNA-cellulose competition assays

Nuclear extracts prepared as for *in vitro* transcription were adjusted to 1 M KCl and applied to a 4.5 ml column of Bio-gel HTP in 5 mM HEPES, pH 7.4, 10% glycerol, 1 M KCl, 1 mM Na₂EDTA, 1 mM 2-mercaptoethanol, 150 µg/ml PMSF to remove contaminating DNA. The column was washed with 15–20 ml of the same buffer, removing 90% of the protein, and then with the same buffer containing 50 mM potassium phosphate to elute receptor, which was concentrated using affinity chromatography (Wright *et al.*, 1983) and passage over Sephadex LH-20 in binding buffer (20 mM HEPES, pH 7.4, 10% glycerol, 100 mM KCl, 2 mM MgCl₂, 70 µM ZnCl₂, 1 mM 2-mercaptoethanol, 150 µg/ml PMSF). Receptors for use in DNA-cellulose assays were then incubated with 5 nM [³H]oestradiol plus or minus 1 µM unlabelled oestradiol for 1 h at room temperature. Each DNA-binding reaction (150 µl in binding buffer) contained 2 µg of calf thymus DNA-cellulose (Alberts and Herrick, 1971), 15–20 fmol of receptor and competitor DNA. After 40 min at room temperature (allowing complete binding), 1 ml of ice-cold binding buffer containing 0.5 mg/ml of BSA, 1 µM testosterone and 1 µM dexamethasone (to suppress non-specific interaction between free [³H]oestradiol and DNA-cellulose) was added. The DNA-cellulose was pelleted, washed twice and counted. Bound receptor was estimated using one aliquot labelled at all sites (5 nM [³H]-oestradiol alone) and another labelled at low-affinity sites (5 nM [³H]-oestradiol plus 1 µM unlabelled oestradiol).

Gel-retardation assays

Each binding reaction (35 µl) contained affinity-purified nuclear extract (3 µg of protein, 30 fmol of receptor), 200 pg (0.15 fmol) of ³²P-end-labelled DNA, 50 ng of tRNA and poly(dI-dC)·poly(dI-dC). After 15 min at room temperature, samples were applied to 4% polyacrylamide gels running in Tris-glycine buffer at 10 V/cm. Preliminary experiments showed that retarded complexes were formed with a receptor:fragment ratio of 200:1 and that with a ratio of 100:1 the interaction was barely discernible.

DNase I footprinting

Binding reactions as used for gel retardation were scaled up 10-fold. Each reaction (350 µl) containing 300 fmol of receptor (native or photoinactivated), 2 ng (1.5 fmol) of end-labelled DNA, 500 ng of tRNA and 100 ng of poly(dI-dC)·poly(dI-dC) in binding buffer and 1 µM oestradiol was incubated for 15 min at room temperature. MgCl₂ was added to 10 mM and CaCl₂ to 5 mM. DNase I (200 ng) was added, and digestion was stopped after 1 min at room temperature by adding excess EDTA and EGTA. The whole of each sample was run on the gel-retardation system. Retarded complexes were located by autoradiography, and the DNA in them was electro-eluted and applied to sequencing gels.

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References

- Alberts,B. and Herrick,G. (1971) *Methods Enzymol.*, **21**, 198–217.
- Corthésy,B., Hipskind,R., Theulaz,I. and Wahli,W. (1988) *Science*, **239**, 1137.
- Everett,M.A., Yeagers,E., Sayre,R.A. and Olson,R.L. (1966) *Photochem. Photobiol.*, **5**, 533–542.
- Gast,P.R. (1960) In *Handbook of Geophysics*. Macmillan, New York, pp. 16–14–16–32.
- Germond,J.-E., ten Heggeler,B., Schubiger,J.-L., Walker,P., Westley,B. and Wahli,W. (1983) *Nucleic Acids Res.*, **11**, 2979–2997.
- Green,S., Kumar,V., Krust,A. and Chambon,P. (1987) In Moudgil,V.K. (ed.), *Recent Advances in Steroid Hormone Action*. De Gruyter, Berlin, pp. 161–183.
- Hatchard,C.G. and Parker,C.A. (1956) *Proc. Roy. Soc.*, **A235**, 518–536.
- Katzenellenbogen,J.A., Carlson,K.E., Johnson,H.J. and Myers,H.N. (1976) *J. Toxicol. Environ. Health, Suppl.*, **1**, 205–230.
- Katzenellenbogen,J.A., Johnson,H.J., Carlson,K.E. and Myers,H.N. (1974) *Biochemistry*, **13**, 2986–2994.
- Kazmaier,N., Bruning,E. and Ryffel,G.U. (1985) *EMBO J.*, **4**, 1261–1266.
- Klein-Hitpass,L., Kaling,M. and Ryffel,G.U. (1988) *J. Mol. Biol.*, **201**, 537–544.
- Klein-Hitpass,L., Tsai,S.Y., Greene,G.L., Clark,J.H., Tsai,M.-J. and O'Malley,B.W. (1989) *Mol. Cell. Biol.*, **9**, 43–49.
- Knuppen,O.H. and Hoppen,H.-O. (1982) *Steroids*, **39**, 667–673.
- Korenman,S.G. (1974) *Endocrinology*, **87**, 1119.
- Kumar,V., Green,S., Staub,A. and Chambon,P. (1986) *EMBO J.*, **5**, 2231–2236.
- Longwell,B. and Wintersteiner,O. (1940) *J. Biol. Chem.*, **133**, 219–229.
- Martinez,E. and Wahli,W. (1989) *EMBO J.*, **8**, 3781–3791.
- Martyr,R.J. and Benisek,W.F. (1975) *J. Biol. Chem.*, **250**, 1218–1222.
- May,F.E.B., Weber,R. and Westley,B.R. (1982) *Nucleic Acids Res.*, **10**, 2791–2807.
- Metzger,D., White,J.H. and Chambon,P. (1988) *Nature*, **334**, 31–36.
- Ogez,J.R., Tivol,W.F. and Benisek,W.F. (1977) *J. Biol. Chem.*, **252**, 6151–6155.
- Rodbard,D. (1973) In O'Malley,B.W. and Means,A.R. (eds), *Receptors for Reproductive Hormones*. Plenum, New York, p. 289.
- Roza,L., Baan,R.A., van der Leun,J.C. and Kligman,L. (1989) *J. Photochem. Photobiol.*, **B3**, 281–287.
- Tora,L., Mullick,A., Metzger,D., Ponglikitmongkol,M., Park,I. and Chambon,P. (1989) *EMBO J.*, **8**, 1981–1986.

- Tsai,S.Y., Tsai,M.-J. and O'Malley,B.W. (1989) *Cell*, **57**, 443–448.
- ten Heggeler-Bordier,B., Hipskind,R., Seiler-Tuyns,A., Martinez,E., Corthésy,B. and Wahli,W. (1987) *EMBO J.*, **6**, 1715–1720.
- Tyrrell,R.M. and Pidoux,M. (1989) *Photochem. Photobiol.*, **49**, 407–412.
- Wahli,W. (1988) *Trends Genet.*, **4**, 227–232.
- Walker,P., Germond,J.-E., Brown-Luedi,M., Givel,F. and Wahli,W. (1984) *Nucleic Acids Res.*, **12**, 8611–8626.
- Wangh,L.J. and Knowland,J. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 3172–3175.
- Waterman,M.L., Adler,S., Nelson,C., Greene,G.L., Evans,R.M. and Rosenfeld,M.G. (1988) *Mol. Endocrinol.*, **2**, 14–21.
- Webster,N.J.G., Green,S., Tasset,D., Ponglikitmongkol,M. and Chambon,P. (1989) *EMBO J.*, **8**, 1441–1446.
- Westley,B.R. and Knowland,J. (1978) *Cell*, **15**, 367–374.
- Westley,B., May,F.E.B., Brown,M.C., Krust,A., Chambon,P., Lippman,M.E. and Rochefort,H. (1984) *J. Biol. Chem.*, **259**, 10030–10035.
- Wright,C.V.E., Wright,S.C. and Knowland,J. (1983) *EMBO J.*, **2**, 973–977.

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