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Elevated UV-B radiation reduces genome stability in plants

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Long-term depletion of the stratospheric ozone layer contributes to an increase in terrestrial solar ultraviolet-B radiation[1–3]. This has deleterious effects on living organisms, such as DNA damage[4,5]. When exposed to elevated ultraviolet-B radiation (UV-B; 280–315 nm), plants display a wide variety of physiological and morphological responses characterized as acclimation and adaptation[6]. Here we show, using special sun simulators, that elevated solar UV-B doses increase the frequency of somatic homologous DNA rearrangements in *Arabidopsis* and tobacco plants. Increases in recombination are accompanied by a strong induction of photolyase and Rad51 gene expression. These genes are putatively involved in major DNA repair pathways, photoreactivation and recombination repair[7,8]. In mutant *Arabidopsis* plants that are deficient in photoreactivating ultraviolet-induced cyclobutane pyrimidine dimers, recombination under elevated UV-B regimes greatly exceeds wild-type levels. Our results show that homologous recombination repair pathways might be involved in eliminating UV-B-induced DNA lesions in plants. Thus, increases in terrestrial solar UV-B radiation as forecasted for the early 21st century may affect genome stability in plants.

The stratospheric ozone layer is the key factor in reducing solar UV-B radiation reaching the Earth's surface[3]. The continuous depletion of the ozone layer will, therefore, be accompanied by an increase in terrestrial UV-B irradiance, especially at wavelengths below 300 nm[1–3,5]. Whereas the impact of UV-B radiation on plant growth, development, physiology and morphology has been studied intensively[9], little is known about its influence on plant genome stability. Homologous recombination is particularly important to plants as their genomes contain large amounts of repeated DNA sequences and highly homologous gene families[10]. In addition, rearrangements between homologous DNA sequences in somatic cells are strongly stimulated by DNA-damaging agents[10].

To obtain a realistic estimate of the long-term impact of increased solar UV-B irradiance on plant genomes, we exposed *Arabidopsis* and tobacco plants over several generations to different levels of elevated UV-B radiation. Genome stability was monitored by assaying homologous recombination in treated plants and their offspring. To simulate the natural photobiological environment we provided a natural spectral balance of photosynthetic active radiation (PAR; 400–700 nm) of 800 μmol m^{-2} s^{-1} and ultraviolet-A radiation (UV-A; 315–400 nm) of 19 W m^{-2}, conditions under which photobiological processes are activated to normal physiological levels[11]. The spectrum was supplemented with four different UV-B regimes[12] which are given as biologically effective (BE) weighted daily UV-B dose normalized at 300 nm[13] (details on irradiation spectra and an alternative weighting are available as Supplementary Information). The 'ambient' UV-B regimes, UV-B1 (2.3 kJ m^{-2} d^{-1}_{BE}) and UV-B2 (6.6 kJ m^{-2} d^{-1}_{BE}), lie in the range of UV-B currently encountered during the growing period in northern mid-latitudes (40° N–60° N) at altitudes up to about 1,000 m above sea level. Two 'high' regimes, UV-B3 (18.6 kJ m^{-2} d^{-1}_{BE}) and UV-B4 (27.1 kJ m^{-2} d^{-1}_{BE}) were used to test the plant's response to strongly elevated UV-B radiation.

The influence of increased UV-B radiation on somatic recombination frequency was analysed using one transgenic *Nicotiana tabacum* line and four independent *Arabidopsis thaliana* lines, carrying one or more copies of a recombination reporter transgene at a single but different locus each. All recombination substrates contain two overlapping parts of the β-glucuronidase gene which are orientated as tandem or inverted repeats and are interrupted by a hygromycin resistance gene. Homologous recombination between these overlapping sequences leads to a complete, functional β-glucuronidase gene. The length of overlap between the two homo-

Table 1 Somatic recombination frequency per genome ($\times 10^{-6}$) in transgenic *Arabidopsis* and tobacco plants

Daily UV-B dose (kJ m^{-2} d^{-1}_{BE})	Line N9	Line A11	Line A211	Line A231	Line A651
2.3 (UV-B1)	11.3 ± 2.1	35.3 ± 4.9	0.6 ± 0.1	0.1 ± 0.0	1.1 ± 0.1
6.6 (UV-B2)	52.3 ± 8.2	58.6 ± 6.2	2.8 ± 0.5	0.2 ± 0.1	2.2 ± 0.3
18.6 (UV-B3)	n.d.	70.9 ± 9.2	5.1 ± 0.7	0.5 ± 0.1	4.4 ± 0.5
27.1 (UV-B4)	83.5 ± 17.1	86.8 ± 13.3	8.4 ± 1.0	0.8 ± 0.2	6.9 ± 0.6

Daily UV-B dose (kJ m^{-2} d^{-1}_{BE})	Line A87	Line A94
2.3	0.5 ± 0.1	0.9 ± 0.1
7.7	1.2 ± 0.2	6.5 ± 1.1
13.4	2.2 ± 0.4	25.1 ± 2.9

	Generation (Line A651) F_0	F_1	F_2
2.3	1.3 ± 0.2	1.3 ± 0.2	1.4 ± 0.3
6.6	2.4 ± 0.3	5.7 ± 0.8	7.7 ± 1.1
18.6	4.9 ± 0.7	12.3 ± 1.8	15.7 ± 2.2

Each value represents the mean of 80–120 plants ± s.e. The structure of the recombination substrates for the different lines has been described[14,15]. In brief, the tobacco line N9 carries four copies of the recombination substrate in indirect orientation, with 566-base pair (bp) homologous overlapping sequence; the *Arabidopsis* line A11 contains one copy of the recombination substrate in direct orientation, with 1,213-bp overlap; the *Arabidopsis* lines A211 (one copy) and A231 (two copies) carry the recombination substrates in direct orientation with 566-bp overlap (P. Swoboda, personal communication), and line A651 one copy in indirect orientation, with 566-bp overlap. n.d., not determined.

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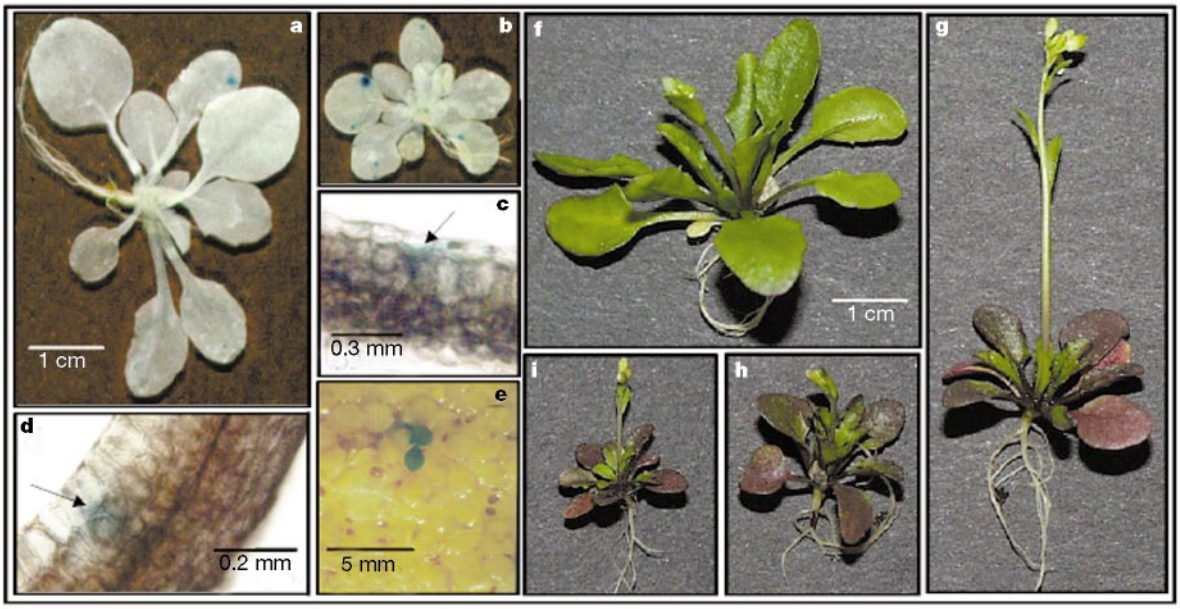

Figure 1 Transgenic *Arabidopsis thaliana* plants (line A11) grown under different daily UV-B doses. **a**, **b**, Visualization by histochemical staining of somatic recombination events in 23-day-old plants exposed to UV-B1 (**a**) and UV-B4 (**b**). The scale is the same for both. **c**, **d**, Sections through stained leaves from plants exposed to UV-B4. Arrows indicate recombination events. **e**, Germinal recombination event in an F_1 population of 7-day-old seedlings grown under UV-B3 regime. The F_0 generation was grown under the identical UV-B3 regime. **f–i**, 23-day-old *Arabidopsis*, germinated and grown under 41 mol m^{-2} d^{-1} photosynthetic active radiation with different daily UV-B doses. **f**, UV-B1 = 2.3 kJ m^{-2} $d^{-1}{}_{BE}$; **g**, UV-B2 = 6.6 kJ m^{-2} $d^{-1}{}_{BE}$; **h**, UV-B3 = 18.6 kJ m^{-2} $d^{-1}{}_{BE}$; and **i**, UV-B4 = 27.1 kJ m^{-2} $d^{-1}{}_{BE}$. Scale is the same for **f–i**.

logous sections and their orientation to each other varies between recombination substrates[14,15] (Table 1). After histochemical staining of plants, recombination events can be visualized and their number and location determined (Fig. 1a–e).

In all the lines tested, the somatic recombination frequency under the current ambient UV-B1 level ranged from $(1.0 \pm 0.2) \times 10^{-7}$ to $(3.5 \pm 0.5) \times 10^{-5}$ (mean ± s.e.) recombination events per genome (Table 1). The large variations between the lines may result from differences in the structure of the recombination substrates, transgene copy number and plant species. Differences in the genomic position of the randomly integrated recombination substrates may also be an important factor influencing both transcriptional activity and recombination frequency[15,16].

Exposure to different levels of elevated UV-B radiation induced morphological changes (Fig. 1f–i) and markedly affected the somatic recombination frequency in all the plant lines tested (Table 1). Under the elevated but still ecologically relevant UV-B2 regime the recombination frequency in tobacco and all *Arabidopsis* lines increased significantly ($P < 0.05$, *t*-test). The recombination frequencies in line N9 rose 4.6-fold, in line A11 1.7-fold, in line A211 4.6-fold and in lines A231 and A651 twofold. With the higher UV-B doses, UV-B3 and UV-B4, recombination was affected even more (up to 8.5-fold increases between UV-B1 and UV-B3 and up to 14-fold increases between UV-B1 and UV-B4). It seems that, in general, the induction of recombination under elevated UV-B radiation is independent of the plant species and line analysed and is probably a genome-wide phenomenon with varying specificity for different loci. The formation and position of UV-induced damage in DNA, as well as the accessibility and activity of DNA repair processes, are influenced largely by chromatin structure, at least in yeast and mammalian cells[17,18]. Different loci in the plant genome may also respond to UV-B stress with different increases in recombination activity.

Table 2 Germinal recombination frequency in *Arabidopsis* plants of line A11

Daily UV-B dose (kJ m^{-2} d^{-1})$_{BE}$	Germinal recombination (events per 250,000 seedlings)
2.3 (experiment 1)	1
2.3 (experiment 2)	2
6.6	4
18.6	10

Increased levels of recombination under elevated UV-B radiation may be an immediate consequence of increased damage and repair of affected DNA. To test whether repair of UV-B-induced DNA damage influences recombination, we crossed the transgenic *Arabidopsis* line A651 with the photolyase deficient line *uvr*2-1 (ref. 19), which lacks photoreactivation repair of cyclobutane pyrimidine dimers, the major UV-B-induced DNA damage. In the resulting progeny line A94, homozygous for *uvr*2-1, the presence of UV-B radiation generally stimulated homologous recombination to a greater extent than in the control progeny line A87, homozygous for *UVR*2-1 (Table 1). In UV-B1, the recombination frequency in line A94 was already about 1.8-fold higher than in line A87. Photolyase-deficient plants were much more sensitive to UV-B radiation than wild-type plants, therefore, we applied slightly reduced regimes. A daily dose of 7.7 kJ m^{-2} $d^{-1}{}_{BE}$ UV-B induced recombination in line A94 5.4 times more strongly than in line A87 and a dose of 13.4 kJ m^{-2} $d^{-1}{}_{BE}$ UV-B increased the recombination frequency up to 11.4 times more efficiently than in line A87. Higher UV-B doses were lethal for photolyase-deficient plants. The absence of cyclobutane pyrimidine dimer-specific photoreactivation repair increased the steady-state concentration of cyclobutane pyrimidine dimers in mutants exposed to elevated UV-B levels, compared with wild-type plants (Fig. 2a). In contrast, the concentration of (6-4) photoproducts was unaffected under elevated UV-B regimes in photolyase mutant plants, as in wild-type plants (data not shown). These data indicate that there may be a strong relationship between cyclobutane pyrimidine dimers in the plants' genomes and homologous recombination, and furthermore that recombination might be involved in cyclobutane pyrimidine dimer repair. Elimination of UV-B-induced DNA lesions, by DNA repair pathways such as nucleotide excision, may create recombinogenic intermediates. Alternatively, or in addition, UV-B-promoted signalling pathways may activate recombination processes by stimulating, for example, transcription of DNA repair genes[20] (see below).

Plants may compensate for deleterious effects of elevated UV-B radiation by mounting various acclimation responses, including accumulation of secondary metabolites and stimulation of DNA repair[6]. The accumulation of UV-B-screening compounds, mainly flavonols, is involved in protecting against UV-B radiation[21,22]. We therefore determined the concentration of quercetin and kaempferol derivatives (Fig. 2b) as well as sinapate esters and anthocyanin compounds in UV-B-treated plant populations. In plants exposed to the UV-B2 level, the production of flavonols was significantly induced ($P < 0.01$, *t*-test) compared with UV-B1-treated plants. Under UV-B3 or UV-B4 conditions, only a weak additional flavonol accumulation could be observed (Fig. 2b). Unlike flavonols, sinapate esters were not induced by elevated UV-B radiation (data not shown). The content of the visible anthocyanins was increased under higher UV-B levels (Fig. 1f–i), although to much lower levels than those of flavonols (data not shown), indicating a minor role for the anthocyanins in absorbing UV-B. Under the anticipated elevated UV-B levels the protection against UV-B radiation afforded by UV-B-screening pigments may therefore be incomplete.

This may, at least in part, be counterbalanced by enhanced repair activity. We compared the repair activities in plants exposed to the different UV-B regimes by examining the steady-state levels of AtPhotolyase II (*PHR*1) and AtRad51 messenger RNA, the latter as a putative marker for recombination repair. Using polymerase chain reaction with reverse transcriptase (RT-PCR), we found that the steady-state mRNAs of both genes were increased under high UV-B doses (Fig. 2c). This suggests that plants grown under elevated UV-B regimes have an increased ability to repair DNA damage. This ability may reduce at least DNA damage resulting in cyclobutane pyrimidine dimers and (6-4) photoproducts to the level of that found in plants exposed to ambient UV-B conditions (Fig. 2a). In animals and yeast, Rad51 is crucial for recombination[8,23] and in plant cells it has been reported to be strongly induced after gamma irradiation[24]. If AtRad51 is involved in recombination repair in plants, enhanced expression of AtRad51 under elevated UV-B levels could result in higher recombination repair activity, which in turn may contribute to increased frequencies of homologous recombination.

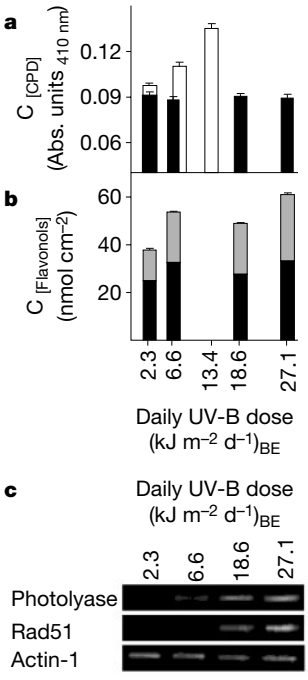

Figure 2 UV-B acclimation responses in *Arabidopsis thaliana* line A651 grown under different daily UV-B doses. **a**, Cyclobutane pyrimidine dimer (CPD) concentrations in wild-type (black bars) and photolyase-deficient plants (white bars). Vertical bars represent s.e. **b**, Flavonol concentrations, measured by quercetin (grey bars) and kaempferol (black bars) derivatives. Vertical bars represent s.d. **c**, Steady state mRNA levels for photolyase II (*PHR*1), AtRad51 protein and AtActin-1 determined by RT-PCR.

Germinal recombination events can permanently alter the genetic composition of subsequent generations and hence contribute to genomic changes in plant populations[25]. Although reproductive organs are generally considered to be well protected from UV-B during both developmental and mature phases, long-term exposure to high UV-B levels may also affect the reproductive tissue (L2 cell layer) of plants and cause DNA damage. Mature pollen grains are potentially very susceptible to UV-B-induced DNA damage during the short period between anther dehiscence and pollen tube penetration into stigma tissues[26]. Indeed, UV-B irradiation of maize pollen can activate cryptic transposable elements[27]. Recombination processes may also be induced under such conditions, affecting the genome stability of future plant generations. We analysed the influence of UV-B radiation on the frequency of germinal recombination in *Arabidopsis* plants. Germinal recombinants are relatively rare in plants and can be the products of meiotic recombination or representatives of somatic homologous interactions[16]. To detect such rare germinal recombinants, we screened large numbers of seedlings of the subsequent generation of plants grown under elevated UV-B regimes. Compared to UV-B1 conditions, UV-B2 regimes increased the number of germinal recombinations in seven-day-old *Arabidopsis* seedlings about four times and UV-B3 regimes up to ten times (Fig. 1e, Table 2). The germination rate and the seed set did not differ between the analysed plant populations (data not shown). Although the numbers of germinal recombination events were small, this preliminary experiment may indicate that elevated UV-B levels can lead not only to an increase in genomic instability of somatic tissue but also to permanent changes in plant populations. Furthermore, the progeny of plants exposed to elevated UV-B exhibited a higher UV-B-induced somatic recombination rate than the parental population. Whereas ambient UV-B1 levels did not stimulate somatic recombination in subsequent plant generations, exposure of populations to UV-B2 and UV-B3 resulted in a marked increase in the recombination frequency in progeny plants (Table 1). When exposed to similar UV-B2 levels as the parental plants, the F_1 progeny showed a 2.4-fold and the F_2 progeny a 3.2-fold higher recombination frequency than F_0 plants. Under UV-B3 regimes the recombination frequencies in the F_1 and the F_2 generation increased by nearly the same factors of 2.5 and 3.2, respectively. Thus, elevated UV-B exposure over several generations may lead to progressive increases in somatic recombination rates as well as to higher numbers of permanently altered plants.

Under natural conditions the terrestrial UV-B irradiance varies strongly depending on the thickness of the UV-screening ozone layer, the solar elevation and meteorological conditions. Therefore, extrapolations from data obtained under experimental, static UV-B conditions to natural, dynamic UV-B field conditions are difficult. Our data, however, allow extrapolations on the entire ecologically relevant UV-B range because all recombination data obtained under the 'high' UV-B regimes (UV-B3, UV-B4) are in line with the results obtained under the currently ecological UV-B range (UV-B1, UV-B2). We believe, therefore, that the increase in solar UV-B radiation, due to a long-term depletion of the stratospheric ozone layer, may influence the genomic stability of plant populations. □

Methods

UV spectra

A combination of four lamp types (metal halide lamps, quartz halogen lamps, blue fluorescent tubes and UV-B fluorescent tubes) was used to obtain a natural balance of simulated global radiation throughout the ultraviolet to infrared spectrum. The short-wave cut-off was shaped by selected borosilicate, soda-lime and acrylic glass sheets[12]. The mean PAR was 800 $\mu mol\, m^{-2}\, s^{-1}$, which corresponds to a daily dose of

~41 mol m^{-2} d^{-1} PAR. The calculations for the UV-B doses were performed with the general plant action spectrum normalized at 300 nm[13]. For comparison, we also provide the UV levels weighted with the action spectrum for dimer formation also normalised at 300 nm (dimer)[28] (for details see Supplementary Information).[11] The ratio (UV-B:PAR) is based on the non-weighted irradiance in the PAR and UV-B ranges. The spectroradiometric measurements were performed with a double monochromator system[12]. Variations of the integrated values were less than 15%. We kept the UV-A irradiance constant at ~19 W m^{-2} (non-weighted value) which corresponds to the moderate PAR value. Under the UV-B1 regime, the simulated UV-B:UV-A:PAR ratio of 1:50:480 (rounded figures) closely approximates an average present-day outdoor value around noon of 1:47:400 in northern mid-latitudes under an ozone column of ~320 Dobson units (typical ratio measured at Neuherberg (48° 41′ N, 11° 41′ E), South Germany, 495 m above sea level).

Growth conditions

Plants were germinated in soil and grown under 800 μmol m^{-2} s^{-1} PAR and different UV-B regimes. All climate parameters (14 h light at 20 °C, 10 h dark at 16 °C, 70% humidity) were identical for all ultraviolet treatments.

Somatic recombination frequency

23-day-old transgenic plants were stained histochemically and the recombination frequencies per genome were calculated[14]. In photolyase-deficient plants the recombination frequency was analysed 18 days after germination.

Germinal recombination frequency

Germinal recombination was determined in *Arabidopsis* seedlings of line A11. The F_0 generation was germinated and grown under UV-B1, UV-B2 or UV-B3 until seeds could be harvested. For each UV-B condition around 250,000 seeds were sown on wet filter paper placed on soil. After seven days exposure to similar UV-B regimes as progenitor plants, the seedlings were histochemically stained and the number of fully stained seedlings determined.

Crossings

Arabidopsis photolyase mutant (*uvr*2-1)[19] was crossed with line A651 and independent lines were generated. F_3-generation lines A94 (homozygous for *uvr*2-1) and A87 (homozygous for *UVR*2-1) are both homozygous for line A651 recombination substrate.

Dimer concentration

CPDs and (6-4) photoproducts were quantified[29] in 23-day-old wild-type plants and 18-day-old photolyase-deficient plants. DNA was extracted from 100 mg plant tissue. CPD- and (6-4) photoproduct-specific monoclonal antibodies were purchased from Kamiya Biomedical Company. Samples were measured in quadruplicate and compared by analysis of variance within each experiment, using the computer program STATISTICA.

Flavonol concentration

Quercetin and kaempferol derivatives were analysed[30] in 23-day-old plants as used in Fig. 2a. Samples were analysed in duplicate or triplicate within each experiment.

Steady state mRNA levels

We used ~100 mg of 23-day-old plants, which were germinated and grown under the indicated conditions, to prepare total RNA using standard protocols. Reverse transcription was performed according to the protocol of the manufacturer (Pharmacia) for RT beads using 1 μg of total RNA. The complementary DNA was diluted 1:100, and 1 μl was used for PCR with specific primers. The primer sequences were for AtPhotolyase II (*PHR*1) (AF053365) 5′-GCCGTCGTTTTCAATCTGTT-3′ and 3′-AATCGATTTGGTGGTGAAGC-5′; for AtRad51 (U43528) 5′-CGTTGAGGAAAGGAAGAGCA-3′ and 3′-GTGGCCAAAAACATC AATCC-5′; and for AtActin-1 (M20016) 5′-AAAGGATGCTTATGTTGGCG-3′ and 3′-AGCCACATACATAGCAGGGG-5′. Products with predicted sizes of 103 base pairs (bp) for AtPhotolyase, 132 bp for AtRad51 and 259 bp for AtActin-1 were obtained. The actin transcript was used as constitutive control. The cDNAs for all tested genes were amplified using hot start PCR under the following conditions: 94 °C for 5 min; followed by 40 cycles for AtRad51, 35 cycles for photolyase and 30 cycles for AtActin-1 of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s; ending with 10 min at 72 °C. Equal loading of each amplified gene sequence was determined by the control AtActin-1 PCR product.

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Structure of the dimerized hormone-binding domain of a guanylyl-cyclase-coupled receptor

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The atrial natriuretic peptide (ANP) hormone is secreted by the heart in response to an increase in blood pressure. ANP exhibits several potent anti-hypertensive actions in the kidney, adrenal gland and vascular system. These actions are induced by hormone binding extracellularly to the ANP receptor[1], thereby activating its intracellular guanylyl cyclase domain for the production of cyclic