

data could be explained if a single message or polypeptide is processed to yield DDBF1 and DDBF2. Alternatively, if two proteins are involved, one protein may bind directly to the damaged DNA and the second protein may bind only as part of a complex with the first protein, in a manner analogous to *uvrA* and *uvrB* proteins in *E. coli*. Then the absence of one protein in XP group E would lead to the disappearance of bands B1 and B2. Definition of the XP group E defect awaits experiments in which the exogenous introduction of a single gene corrects the DNA repair defect in intact cells.

The two individuals from XP group E suffered from skin disease less severe than that seen for several other complementation groups (13). It is possible that their cells contained low levels of DDBF1 and DDBF2 sufficient to ameliorate the severity of disease but too low to be detected in these experiments. Alternatively, the factors may have been completely absent, and other mechanisms in the cell permitted partial recognition and removal of DNA damage.

In conclusion, human cells contain nuclear protein factors that bind specifically to damaged DNA and are absent from XP group E cells. These data are strong evidence that the protein factors participate in a versatile DNA repair system in humans. In addition, our results suggest that probes made from physically altered DNA might be useful for detecting other proteins of biological interest.

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Mammalian *ZFY* Sequences Exist in Reptiles Regardless of Sex-Determining Mechanism

J. J. BULL, DAVID M. HILLIS, SHYRIL O'STEEN

In some reptiles, egg incubation temperature determines whether the embryo hatches as male or female; in others, sex chromosomes determine sex. A cloned gene (*ZFY*) representing the putative testis-determining factor in mammals was hybridized to genomic DNA of reptiles with sex chromosomes and to DNA of reptiles with temperature-dependent sex determination. No sex differences in hybridization patterns were observed. Hybridization of *ZFY* to polyadenylated RNA indicates that reptilian versions of this gene are expressed in embryos of both sexes during the temperature-sensitive period. If these highly conserved sequences are important in reptilian sex determination, then temperature-dependent and genotypic sex determination may have a similar molecular basis. For reptiles with XX/XY or ZZ/ZW systems, the absence of sex differences in hybridization patterns raises the question of whether the *ZFY* sequences reside on their sex chromosomes.

A DNA SEQUENCE ENCODING A "zinc finger" protein was recently cloned from the region of the human Y chromosome that causes male development, and this gene is suspected of being the testis-determining factor (1). Similar sequences are present on both the X and Y chromosomes of eutherian mammals, but when the cloned gene (*ZFY*) is hybridized to restriction endonuclease-digested genomic DNA, males reveal two bands of hybridization versus a single band in females, indicating that this gene or its flanking sequences have diverged somewhat between the X and Y. A similar gene is also present in chickens but shows no male-female difference in hybridization pattern (1). We have examined the other major groups of amniotes (squamates, turtles, and crocodilians) for the presence of a homolog to the mammalian *ZFY*. These groups differ from both mammals and birds in that all contain species in which sex is determined by embryonic incubation temperature rather than genotype; squamates and turtles also contain species with sex chromosome systems (2, 3).

Genomic DNA was extracted from reptiles that have different sex-determining mechanisms and from one species of bird (4). The DNA was cut with restriction enzymes, separated by electrophoresis, and transferred to nylon membranes by standard DNA blotting procedures. The blots were

hybridized with probe pDPI007 (5), which encodes human *ZFY* (6). All reptiles and the bird that we studied exhibited hybridization with this probe, typically resulting in a single major band of 2 to 8 kb, but some species exhibited an additional one or two weak bands (Table 1 and Fig. 1). Hybridization was observed in reptiles with sex chromosome systems and in reptiles with temperature-dependent sex determination, so there is no obvious difference between these two

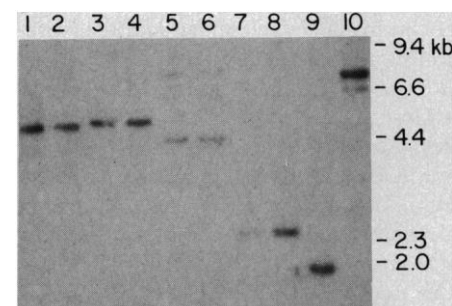


Fig. 1. Genomic DNA (3 to 10 μ g per lane) digested with Pst I. The DNA in lanes 1 to 8 is from reptile species known or presumed to have sex chromosomes; DNA in lanes 9 and 10 is from species with temperature-dependent sex determination. (Lanes 1 and 2) Water moccasin snake (*Agkistrodon piscivorus*), male and female, respectively; (lanes 3 and 4) spiny softshell turtle (*Trionyx spiniferus*), male and female; (lanes 5 and 6) canyon lizard (*Sceloporus merriami*), male and female; (lanes 7 and 8) marbled whiptail lizard (*Cnemidophorus tigris*), male and female; (lane 9) leopard gecko (*Eublepharis macularius*), male; (lane 10) alligator (*Alligator mississippiensis*), male. Molecular size markers are indicated on the right.

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Department of Zoology, University of Texas, Austin 78712.

Table 1. Reptiles exhibiting hybridization to *ZFY* sequences. No sex differences in hybridization patterns were evident. Symbols M and F refer to males and females, respectively; XX/XY and ZZ/ZW systems were originally identified from cytological heteromorphisms (2, 3, 10). Genotypic sex determination is assumed for *Trionyx* because hatching sex ratios are not affected by temperature, but sex chromosomes have not been identified.

Taxon	Sex-determining mechanism	Procedures
Lizards		
<i>Cnemidophorus tigris</i>	XX/XY	1M, 1F studied with Pst I, Hind III
<i>Uta palmeri</i>	XX/XY	1M, 1F; Eco RI, Hind III
<i>Sceloporus merriami</i>	XX/XY	1M, 2F; Pst I, Hind III, Eco RI
<i>Eublepharis macularius</i>	Temperature	1M, 1F; Eco RI; Pst I, Hind III used only on the male
Snakes		
<i>Agkistrodon piscivorus</i>	ZW/ZZ	1M, 1F; Pst I, Hind III, Eco RI
<i>Pituophis melanoleucus</i>	ZW/ZZ	1M, 1F; Eco RI, Hind III
Turtles		
<i>Chelydra serpentina</i>	Temperature	1M; Pst I, Hind III
<i>Trachemys scripta</i>	Temperature	1M; Pst I, Hind III
<i>Trionyx spiniferus</i>	Genotype	1M, 1F; Pst I, Hind III
Crocodylians		
<i>Alligator mississippiensis</i>	Temperature	1M; Pst I, Hind III, Eco RI
Birds		
<i>Nymphicus hollandicus</i>	ZW/ZZ	1M, 1F; Eco RI

mechanisms regarding the presence of *ZFY*-like sequences.

The absence of male-female differences in hybridization patterns has led us to consider whether *ZFY* is located on the reptilian X and Y chromosomes. (Although the notation XX/XY is sometimes reserved for male heterogamety and ZZ/ZW for female heterogamety, we henceforth subsume male and female heterogamety under XX/XY.) By itself, the similar hybridization patterns between males and females means only that the length of cut DNA containing *ZFY*-like sequences is approximately the same between males and females, not that the sequences are the same. Yet, some of the reptiles studied possess highly differentiated sex chromosomes of remote ancestry (within birds or snakes). If a reptilian version of *ZFY* was the primary sex-determining locus in those species, its X-linked and Y-linked alleles would be expected to diverge over time and accumulate restriction fragment length differences, owing to the lack of recombination between the sex-determining segments of the X and Y.

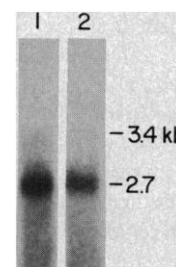
In fact, the data reveal two instances in which a pair of species sharing homologous sex chromosomes exhibit species differences but not sex differences in the restriction fragment lengths hybridizing to the probe [the two snakes, and the chicken from reference (1) compared to the bird used in our study] (7). That restriction fragment lengths have diverged between species but not between the sexes suggest that the *ZFY*-like sequences are autosomal or pseudoautosomal. The data likewise do not appear to support a hypothesis of X-only linkage, absent from the Y (1). An autosomal location

of *ZFY* in reptiles would not preclude a major role in sex determination: most of the major sex determining genes of *Drosophila* and *Caenorhabditis* are autosomal, being expressed downstream of the X-linked, primary sex-determining loci (8).

A reptilian gene hybridizing to pDP1007 was expressed in embryos during the temperature-sensitive period. Polyadenylated RNA extracted from embryos of snapping turtles and slider turtles (both species with temperature-dependent sex determination) was blotted and hybridized with the probe (9). In both species, a strong band was observed at 2.7 kb from embryos that had been kept at male- and female-producing temperatures during the temperature-sensitive period (Fig. 2). There is thus not an absolute difference in expression of reptilian *ZFY* between male-destined and female-destined embryos, although quantitative differences have not been excluded.

Hybridization of this mammalian probe to squamates, crocodylians, and turtles suggests that these sequences are highly conserved. Therefore, if this gene does have a major regulatory role in reptilian sex determination, the molecular basis of temperature-dependent mechanisms and sex chromosome systems may be largely the same. This conclusion, which remains to be demonstrated, would help resolve a dilemma concerning the evolution of reptilian sex determination. The failure to observe an obvious selective advantage of temperature-dependent sex determination over chromosomal sex determination has led to the suggestion that the temperature-dependent systems may lack the genetic variation to evolve sex chromosome systems (3). If the molecu-

Fig. 2. Polyadenylated RNA (7 µg per lane) of turtles hybridized with pDP1007. (Lane 1) Red-eared slider (*Trachemys scripta*) at embryo-stage 18 that was incubated at 31°C, a female-producing temperature. (Lane 2) Snapping turtle (*Chelydra serpentina*) at embryo-stage 19, that was incubated at 25°C, a male-producing temperature. Embryos of each species incubated at temperatures producing the opposite sex of that illustrated exhibit similar bands as those shown here.



lar basis of these two mechanisms were shown to be largely identical, it could no longer be argued that temperature-dependent mechanisms were merely incapable of giving rise to XX/XY mechanisms.

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5. Purified pDP1007 was labeled with ³²P by nick translation as described by P. W. J. Rigby, M. Dieckmann, C. Rhodes, P. Berg, *J. Mol. Biol.* **113**, 237 (1977). Blots were hybridized with *Escherichia coli* carrier DNA for 18 hours at 65°C in 5× SSC, 0.5% SDS, 5× Denhardt's solution, and 2.5 mM KH₂PO₄. Two washes were performed for 15 min in 2× SSC, 0.2% SDS at 37°C, and two additional washes were conducted for 1 hour in 1× SSC, 0.1% SDS at 55°C.
6. To verify that the probe we received was pDP1007, we hybridized it to human male DNA digested with Eco RI and observed bands at the sites described (1).
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- 25°C, followed by two washes for 30 min in 1× SSC, 0.1% SDS at 50°C. After exposure to film, the blot was washed and rehybridized with an actin probe, which indicated that the lanes (in Fig. 2) contained similar quantities of nondegraded RNA.
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In Vivo Administration of Anti-CD3 Prevents Malignant Progressor Tumor Growth

JOSHUA D. I. ELLENHORN, RAPHAEL HIRSCH, HANS SCHREIBER, JEFFREY A. BLUESTONE*

Malignant progressor tumors are only weakly immunogenic and can evade host recognition and rejection. One approach to therapy involves activation of the host antitumor cellular effector mechanisms. Since monoclonal antibodies to CD3 (anti-CD3) can activate T cells in vitro, an attempt was made to determine if tumor immunity could be achieved by the administration of anti-CD3 in vivo. T lymphocytes from mice injected with anti-CD3 showed increased interleukin-2 receptor (IL-2R) expression, increased proliferation to recombinant IL-2 (rIL-2), and enhanced reactivity in both an allogeneic mixed lymphocyte reaction and a mixed lymphocyte tumor culture. Malignant tumor growth in treated mice was also examined. The anti-CD3 treatment prevented tumor outgrowth that would have killed untreated animals and also stimulated an in vivo response against a malignant progressor tumor providing lasting tumor immunity.

MONOCLONAL ANTIBODIES (MABS) to T lymphocyte antigens have been used to suppress immune responses in vivo and in vitro by blocking T cell receptor-mediated antigen recognition (1). This property has been exploited in the clinical setting to prevent and reverse organ transplant rejection (2). In addition to their immunosuppressive properties, these MABS can activate resting T cells in vitro. This suggests that they might be efficacious in augmenting immune responsiveness in a clinical setting. We initially observed that T cells become activated in vivo within hours after treatment with anti-CD3. However, under the conditions used, the stimulatory effects were transient and ultimately led to long-term immune dysfunction. We have therefore determined the optimal conditions to facilitate specific beneficial biologic responses with the use of anti-CD3 as an immunostimulatory reagent.

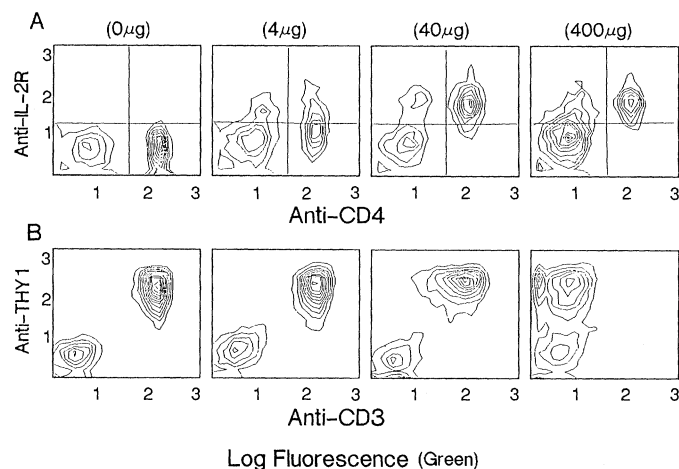
Intravenous administration of 400 µg of the anti-CD3 in vivo has previously been shown to activate T cells (3). This high dose, however, ultimately results in suppression of

T cell function as demonstrated by inhibition of mixed lymphocyte reaction (MLR), cell-mediated lympholysis (CML), and transplantation responses (1). In an attempt to separate these two seemingly contradictory effects of anti-CD3, we examined the

effects of lower MAB doses on T cell activation. Mice were given different doses of anti-CD3, and their lymph node and spleen cells were examined for IL-2R expression 18 hours later by flow cytometry (FCM) (Fig. 1). The IL-2R expression was enhanced at the three doses tested (4 µg, 40 µg, and 400 µg) and plateaued at the 40-µg dose. In other experiments maximal IL-2R expression was found at the 400-µg dose. When the same lymphoid cells were incubated in media containing human rIL-2, their proliferation was enhanced in proportion to their IL-2R expression (Fig. 2). This proliferation was not the result of the carry-over of injected anti-CD3 from the mouse to the culture media, since the addition of MAB 2.4G2 [anti-Fc receptor (FcR)], to the culture media did not affect the proliferative response (4). By comparison, the addition of 2.4G2 to control cells incubated with IL-2 and anti-CD3 eliminated the FcR-dependent proliferative response.

The immunosuppression which results from a dose of 400 µg of anti-CD3 is the result of T cell depletion, T cell receptor (TCR) blockade, and modulation of the TCR complex (1). The net result is that the amount of cell surface CD3 available to react with antigen is decreased, rendering the T cells unable to respond to antigenic stimuli because efficient antigen-specific activation depends on the presence of intact TCR. Therefore, the quantity of available CD3 on lymph node cells from control mice and those treated with various doses of anti-CD3 was examined 18 hours after treatment. Cells were stained with fluorescein

Fig. 1. Flow cytometry of peripheral lymph node cells from anti-CD3-treated C3H mice. Two-color FCM from control, 4, 40, and 400 µg of anti-CD3 treated animals are displayed as contour plots on a logarithmic scale. Intensity of green FITC fluorescence is plotted along the x-axis and red (B-phycoerythrin) fluorescence is plotted along the y-axis. (A) Anti-CD4 staining on the x-axis and anti-IL2R staining on the y-axis. (B) Anti-CD3 staining on the x-axis and anti-Thy-1 staining on the y-axis. C3H/HeN MTV⁻ mice (Frederick Cancer Research Foundation, Frederick, Maryland) were housed and cared for in accordance with the guidelines of the University of Chicago Animal Research Committee. The animals were killed 18 hours after intravenous injection of purified anti-CD3 (MAB 145-2C11) that was grown and purified as described (1). Femoral, axillary, and mesenteric lymph nodes were removed and dissociated into a single-cell suspension and FCM analysis was performed (9). Cells were stained with FITC-anti-CD3 or FITC-anti-CD4 (MAB GK1.5) (Becton Dickinson), and biotin-conjugated anti-IL-2R (MAB 3C7) or biotin-conjugated MAB to Thy-1.2 (Becton Dickinson), then counterstained with B-phycoerythrin-conjugated egg white avidin (Jackson Immuno Research Laboratories).



J. D. I. Ellenhorn, H. Schreiber, J. A. Bluestone, University of Chicago, Chicago, IL 60637.
R. Hirsch, National Cancer Institute, Bethesda, MD 20892.

*To whom correspondence should be addressed.