magnitude over the previous state of the art for amino acid analysis; the best previous amino acid detection limits cluster around 10^{-16} mol for both fluorescence (*11*) and thermo-optical absorbance (*12*) detection. The present detection limits were obtained with a derivatizing reagent that may be used in a modified Edman degradation scheme (*1*). State-of-the-art detection limits with the conventional Edman degradation reagent, phenylisothiocyanate, fall near 10^{-12} mol (*13, 14*), and earlier work with FITC–amino acids has produced detection limits near 10^{-14} mol (*14*).

At present, there is much interest in the sequence determination of minute quantities of proteins (*14*). Because FITC has been used as a modified Edman degradation reagent, it is, in principle, possible to use this reagent to determine the amino acid sequence of low and subattomole quantities of proteins. However, extrapolation of current technology by six orders of magnitude is fraught with difficulty. Before attomole quantities of proteins can be sequenced, at least two significant issues must be addressed. First, the sample produced by the sequenator must be transferred to the electrophoresis column. Conventional sequenators produced sample volumes of a few microliters, whereas it is necessary to use samples of nanoliter or smaller volume in capillary zone electrophoresis. Second, reagent purity must be drastically improved to minimize the reagent blank. The first problem might be avoided by concentrating the amino acid before it is introduced into the separation capillary. Unfortunately, any technique that concentrates the sample also concentrates impurities. Instead, it may be profitable to redesign the solid-phase sequenator to match the volume requirements of the electrophoresis separation. Miniaturization of the sequenator offers several advantages. For example, preliminary concentration of the sample is not necessary, because the sequenator would produce samples matched in size to the requirements of the electrophoresis. Also, miniaturization of the sequenator would result in a proportional decrease in the volume of reagent required for the Edman degradation steps; because only a few nanoliters of reagent might be required for the degradation, costly and very high purity reagents can be used. The development of this miniaturized sequenator will require significant attention to detail to avoid sample loss and contamination. However, techniques developed for the manipulation of minute samples in capillary chromatography, electrophoresis, and flow injection analysis should provide a useful guide in the development of this attomole sequenator.

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Xeroderma Pigmentosum Group E Cells Lack a Nuclear Factor That Binds to Damaged DNA

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The disease xeroderma pigmentosum is characterized by deficient repair of damaged DNA. Fusions of cells from different patients have defined nine genetic complementation groups (A through I), implying that DNA repair in humans involves multiple gene products. In this report, an extension of the gel electrophoresis binding assay was used to identify at least one nuclear factor that (i) bound to DNA damaged by ultraviolet radiation or the antitumor drug cisplatin, but (ii) was notably absent in cells from complementation group E. Therefore, the factor appears to participate in a versatile DNA repair pathway at the stage of binding and recognition.

INDIVIDUALS HOMOZYGOUS FOR XERODERMA pigmentosum (XP) are hypersensitive to ultraviolet (UV) radiation and have a high incidence of skin cancers (*1*). XP cells are defective in the repair of damaged DNA containing UV-induced pyrimidine dimers. So far, the molecular basis for the XP defect has remained undefined. By contrast, the uvrABC endonuclease system in *Escherichia coli*, which catalyzes the excision repair of pyrimidine dimers, is well characterized. UvrA protein binds to UV-irradiated DNA by itself (*2*), but uvrB protein does not (*3*). However, both proteins together form a stable complex of uvrA, uvrB, and DNA. UvrC protein then catalyzes the enzymatic nicking of the damaged DNA. Motivated by the possibility that proteins analogous to uvrA and uvrB might be found in human cell extracts, we extended the gel electrophoresis binding assay (*4*) to the identification of proteins that bind to damaged DNA rather than to specific DNA sequences.

XP cells are defective in the repair of many forms of DNA damage in addition to UV damage. In particular, they are defective in the repair of transfected plasmid DNA that has been cross-linked in vitro by the antitumor drug cisplatin, cis-$Pt(NH_3)_2Cl_2$ (*5*). To find proteins that participate in the DNA repair system defined by the XP mutations, we searched for factors that would bind specifically to DNA damaged by either UV radiation or cisplatin.

DNA probes f65 and f103 were constructed as substrates for UV- and cisplatin-induced damage, respectively (Fig. 1). Fragment f65 contained a string of eight consecutive thymine residues that was a target for UV-induced thymine dimers. Fragment f103 contained a string of 14 consecutive guanine residues as a target for cisplatin, which shows a preference for forming intrastrand cross-links at the N7 position of adjacent guanine residues (*6*).

Nuclear extracts were prepared from HeLa cells by methods previously used to

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identify mammalian transcription factors (7). The extracts were incubated with probe DNA in the presence of the alternating copolymer poly(dI-dC) · poly(dI-dC) to mask nonspecific binding.

The formation of protein-DNA complexes with UV-irradiated probe could be either sequence specific or damage specific. To distinguish between these possibilities, we incubated nuclear extract with both UV-irradiated and intact f65 probe DNA. With intact probe DNA, multiple bands of retarded mobility appeared. However, unlabeled f65 DNA competed efficiently for the binding activity (Fig. 2A, lanes 1 to 5). Thus, binding activity to undamaged f65 DNA was specific for DNA sequence. By contrast, when the probe DNA was UV-irradiated, we observed two major bands, B1 and B2, and one minor band, B3, which persisted in the presence of unlabeled f65 DNA (Fig. 2A, lanes 6 to 10). Binding was protein-dependent, since it was sensitive to treatment of the extract with Pronase but insensitive to ribonuclease A. Thus, UV-irradiated DNA allowed the formation of protein-DNA complexes that were independent of DNA sequence.

To study the specificity of the binding activity for damaged DNA, we incubated UV-damaged f65 probe DNA with nuclear extract in the presence of unlabeled DNA of heterogeneous sequence from salmon sperm. In band B3, both damaged and undamaged DNA competed poorly for binding when the gel was exposed for longer periods. Thus band B3 represents a protein-DNA complex that is not specific for damaged DNA. However, in bands B1 and B2, intact DNA competed poorly for binding whereas UV-irradiated and cisplatin cross-linked DNA competed 10 to 100 times more effectively (Fig. 2B). Therefore, bands B1 and B2 represent one or more factors that bind specifically to damaged DNA.

A second probe produced complexes with the same characteristics of bands B1 and B2. Probe f148 was the 148-bp Hind III–Pvu II fragment from the 5′ flanking and coding region of the bacterial chloramphenicol acetyltransferase gene (*8*). Intact f148 probe did not form any specific protein-DNA complexes on incubation with HeLa extract. When f148 probe was UV-irradiated, two bands appeared at positions on the gel slightly higher than bands B1 and B2. This is noteworthy, since the mobility of a protein-DNA complex is relatively insensitive to the length of the DNA probe flanking the protein binding site (*9*). UV-irradiated salmon sperm DNA competed efficiently for binding, whereas intact salmon sperm DNA did not. Furthermore, UV-irradiated f148 DNA competed efficiently for the binding activity to UV-irradiated f65 probe in bands B1 and B2. Thus, the factors in bands B1 and B2 formed complexes with damaged DNA probes independent of probe sequence.

At least two different factors with different relative affinities for damaged DNA appear to be represented by bands B1 and B2. Band B1 disappeared with 2000 ng of undamaged salmon sperm DNA and 200 ng of UV-damaged DNA (Fig. 2B). Thus, the factor in band B1 has an affinity for UV-damaged DNA that is only ten times greater than its affinity for intact DNA. On the other hand, band B2 was still present when 2000 ng of undamaged DNA was added, but disappeared with only 20 ng of UV-damaged DNA. Thus, the factor in band B2 has an affinity for UV-damaged DNA 100 times greater than for intact DNA. We designate the factors in bands B1 and B2 as damaged DNA binding factors DDBF1 and DDBF2, respectively.

The pattern of binding to the UV-damaged probe was the same for UV doses from 600 to 9000 J/m^2, with a broad peak in binding activity from 4000 to 8000 J/m^2. Doses above 4000 J/m^2 on pSV2gpt DNA will convert approximately 6% of the thymine residues to cyclobutane pyrimidine dimers (*10*). Applying the 6% estimate to the DNA fragment f65, which contains 30 thymines, yields an average of 1.8 thymine-containing cyclobutane dimers per DNA molecule. The actual number of lesions was probably somewhat higher, since regions of consecutive thymines form cyclobutane dimers at a higher rate (*11*). Other photoproducts, such as (6-4) pyrimidine dimers and pyrimidine hydrates must be present at much lower levels. Since the incubation of nuclear extracts with UV-irradiated f65 probe could result in the shifted mobility of much of the labeled probe to bands B1 and

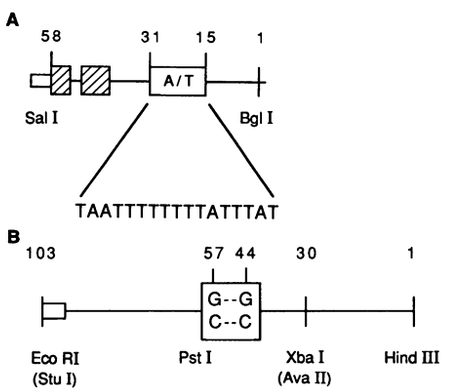

Fig. 1. DNA probes for detection of damaged DNA binding proteins. (**A**) The 65-bp DNA fragment f65 was generated by Sal I and Bgl I cleavage of the plasmid pS-288 (*14*), and contained 58 bp from the simian virus 40 (SV40) promoter-enhancer region. The A/T-rich region was a target for generation of UV radiation–induced thymine dimers. The cross-hatched regions indicate the GC boxes from SV40. (**B**) The 103-bp DNA fragment f103 contains a 14-bp string of dG · dC as a target for cisplatin intrastrand cross-links. Ends generated by Stu I and Ava II cleavage of pCD-α-globin (*15*) were filled in with DNA polymerase I large fragment, ligated to Eco RI and Xba I linkers, respectively, and cloned into pUC19. The fragment was then generated by cleavage with Eco RI and Hind III.

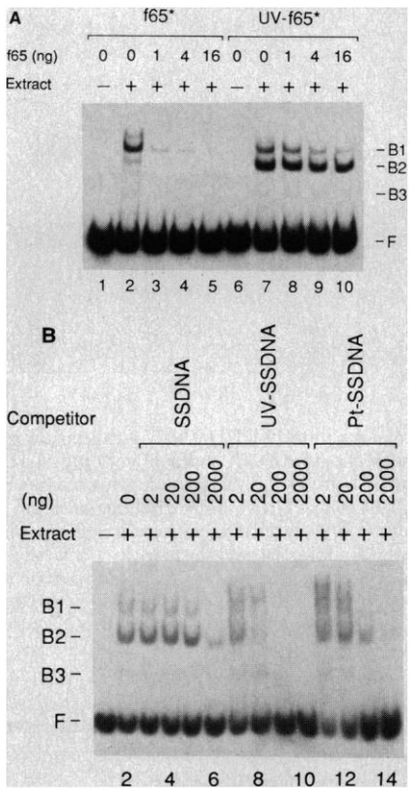

Fig. 2. Binding activity in HeLa extracts specific for damaged DNA. (**A**) Sequence-specific binding identified by competition with f65 DNA. The DNA fragment f65 was gel-purified, end-labeled with DNA polymerase I large fragment, and either left intact (lanes 1 to 5) or irradiated with 9000 J/m^2 from a UV germicidal lamp (lanes 6 to 10). Probe DNA (0.2 ng) was incubated with 2 μg of HeLa nuclear extract as previously described (*16*) in the presence of unlabeled f65 DNA and resolved by electrophoresis in a 5% polyacrylamide gel in TBE buffer (89 m*M* tris, *p*H 8; 89 m*M* borate; 2 m*M* EDTA). F denotes the position of the free probe. Unless otherwise specified, this and subsequent experiments included 1 μg of poly(dI-dC) · poly(dI-dC) (*9*). (**B**) Damaged DNA-specific binding identified by competition with salmon sperm DNA. UV-irradiated probe DNA was incubated with 3 μg of HeLa nuclear extract in the presence of salmon sperm DNA (SSDNA) that was undamaged (lanes 3 to 6), UV-irradiated (lanes 7 to 10), or cross-linked with cisplatin (lanes 11 to 14). Cisplatin binding was at the level of 6 adducts per 100 nucleotides. This gel was run in low ionic strength buffer (6.7 m*M* tris, *p*H 7.9; 3.3 m*M* sodium acetate; 1 m*M* EDTA) (*16*) to demonstrate that different electrophoresis conditions do not lead to a different pattern of bands.

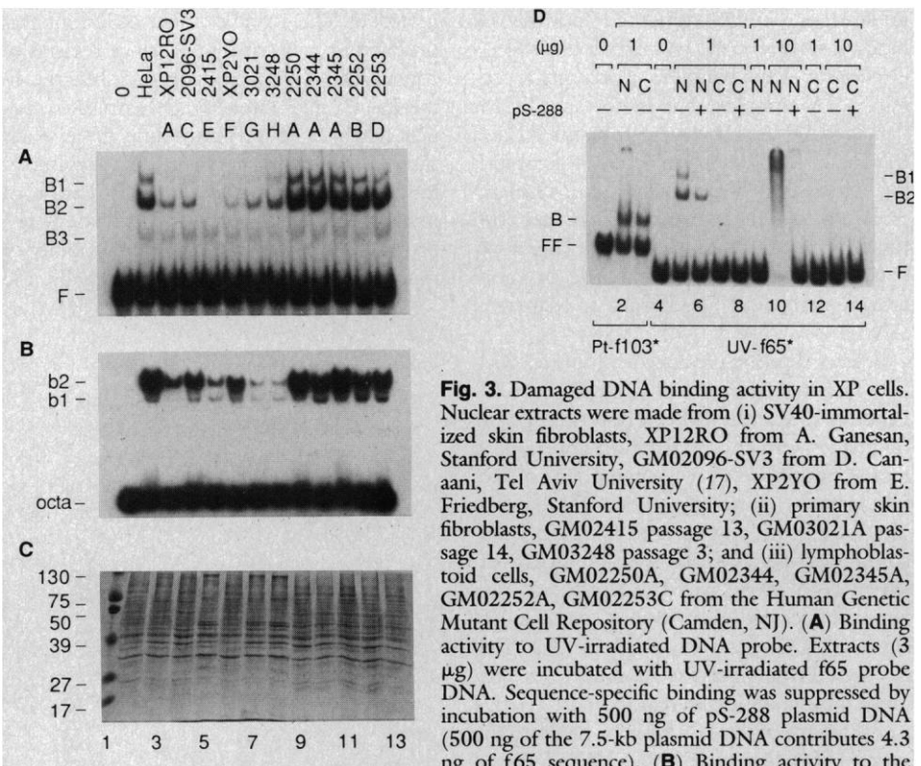

Fig. 3. Damaged DNA binding activity in XP cells. Nuclear extracts were made from (i) SV40-immortalized skin fibroblasts, XP12RO from A. Ganesan, Stanford University, GM02096-SV3 from D. Canaani, Tel Aviv University (*17*), XP2YO from E. Friedberg, Stanford University; (ii) primary skin fibroblasts, GM02415 passage 13, GM03021A passage 14, GM03248 passage 3; and (iii) lymphoblastoid cells, GM02250A, GM02344, GM02345A, GM02252A, GM02253C from the Human Genetic Mutant Cell Repository (Camden, NJ). (**A**) Binding activity to UV-irradiated DNA probe. Extracts (3 μg) were incubated with UV-irradiated f65 probe DNA. Sequence-specific binding was suppressed by incubation with 500 ng of pS-288 plasmid DNA (500 ng of the 7.5-kb plasmid DNA contributes 4.3 ng of f65 sequence). (**B**) Binding activity to the octameric motif ATTTGCAT. Extracts (6 μg) were incubated with 30 pg of the end-labeled 20-bp double-stranded DNA fragment with the sequence TCTAGATGATTTGCATTCTA. Electrophoresis was done in 5% polyacrylamide with tris-glycine buffer (50 m*M* tris, *p*H 8.5; 380 m*M* glycine and 2 m*M* EDTA). (**C**) Abundant nuclear proteins. Extracts (6 μg) were resolved by a modified Laemmli method (*18*) in a 10% polyacrylamide resolving gel and then stained with Coomassie blue. Lane 1 contains molecular weight markers (in kilodaltons) (Bio-Rad). (**D**) Nuclear localization of damaged DNA binding activity. Nuclear (N) and cytoplasmic (C) fractions (7) from HeLa (lanes 2 to 8) and XP group E (lanes 9 to 14) cells were incubated with cisplatin cross-linked probe or UV-irradiated probe, in the presence of 0.5 μg of poly(dI-dC) · poly(dI-dC) with or without 1 μg of pS-288 DNA.

B2 (Fig. 3A, lanes 9 to 13), the observed binding activity was probably specific for cyclobutane pyrimidine dimers.

The amount of binding activity for a given amount of probe DNA can be used to estimate a lower limit for the abundance of the binding factors. If one assumes a 1:1 binding stoichiometry, there are a minimum of about 10,000 molecules of DDBF2 and about 3,000 molecules of DDBF1 per HeLa cell. The actual abundances are probably somewhat higher, since the binding assay does not detect molecules that are bound to the alternating copolymer.

Next, extracts made from cells of the eight complementation groups, A through H, were surveyed for binding activity. Bands B1 and B2 were not detectable in cells from complementation group E. The presence of band B3 was an internal control for the presence of active nuclear extract. However, cells from groups A, B, C, D, F, G, and H contained easily detectable bands B1 and B2 (Fig. 3A). Among different cell types, the factors were more abundant in HeLa cells and lymphoblastoid cells than in skin fibroblasts. The absence of specific binding activity for damaged DNA in group E cells could be corrected by the addition of extracts from groups A, B, C, D, F, G, or H. This rules out the possibility that group E cells contain a factor that inhibits binding activity.

The extract from these XP group E cells appeared normal for other binding activity. For example, they contained factors that bound to an oligonucleotide containing the conserved octameric motif ATTTGCAT (*9, 12*), resulting in two complexes, b1 and b2 (Fig. 3B). Binding activity was present in all cells examined, although the amount varied roughly with cell type.

The XP group E extract also appeared normal for a large number of other proteins. When extracts were analyzed by Coomassie blue stain after electrophoresis in a 5% SDS-polyacrylamide gel (Fig. 3C), minor variations were observed between cell types, but no variations were detectable within each cell type. In particular, group E extract contained the same profile of proteins as the other two primary skin fibroblast lines, 3021 and 3248.

To exclude the possibility that binding activity in XP group E cells might be extracted from nuclei at a slightly higher salt concentration, the procedure was repeated at both 0.42*M* NaCl (the usual concentration) and 0.5*M* NaCl. No detectable binding activity in bands B1 and B2 was observed at either salt concentration. Furthermore, extracts were prepared from a second group E cell line, GM02450D, lymphoblastoid cells from an affected second cousin of the first group E individual. Again, no damage-specific binding activity was observed, even though lymphoblastoid cells generally contain higher levels of such activity. Extracts from both XP group E individuals also failed to show binding activity to UV-irradiated f148 probe. Thus, extracts from XP group E cells prepared on four independent occasions consistently failed to show damage-specific binding activity.

In HeLa cells, the damage-specific binding activity was strongly localized in the nucleus and virtually absent in the cytoplasm (Fig. 3D, lanes 4 to 8). The defect in XP group E cells was not the result of a failure of protein transport to the nucleus, since binding activity was absent in both nuclear and cytoplasmic extracts (Fig. 3D, lanes 9 to 14).

Because competition experiments indicated that DDBF1 and DDBF2 have specificity for cisplatin cross-linked DNA, we performed experiments using labeled cisplatin cross-linked DNA probe, Pt-f103*. In the presence of HeLa extract, the free probe FF was shifted in mobility to band B (Fig. 3D, lanes 1 to 3). Binding activity was sensitive to Pronase and resistant to ribonuclease A. Competition with unlabeled intact f103 DNA did not affect binding, whereas competition with cisplatin cross-linked DNA from either f103 or salmon sperm abolished binding to the probe. Therefore, binding to cisplatin cross-linked DNA was protein-dependent, specific for cisplatin adducts, and independent of DNA sequence. However, the binding activity in band B cannot be due to DDBF1 or DDBF2, since it (i) persisted in the presence of unlabeled UV-damaged DNA, (ii) was present in the extracts from the eight complementation groups A through H, and (iii) was observed in both nuclear and cytoplasmic cell fractions (Fig. 3D, lanes 1 to 3). It is likely that, in crude extracts, the abundance of the factor in band B and steric interference prevent binding by DDBF1 and DDBF2. Direct demonstration of binding by DDBF1 and DDBF2 may require chromatographic fractionation of the extract.

The defect in XP group E cells is yet to be defined. It is unlikely that two distinct proteins are absent, since inheritance is autosomal recessive and the genetic defect is presumably at both alleles of a single locus. The

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

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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 pDP1007 (*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

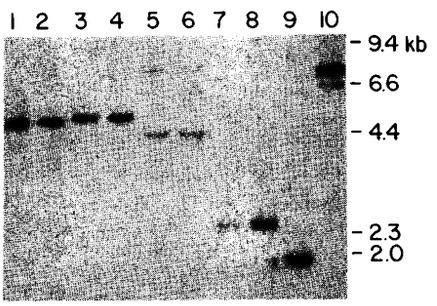

Fig. 1. Genomic DNA (3 to 10 μg per lane) digested with Pst I. The DNA in lanes 1 to 8 is from reptile species known or presumed to have sex chromosomes; DNA in lanes 9 and 10 is from species with temperature-dependent sex determination. (Lanes 1 and 2) Water moccasin snake (*Agkistrodon piscivorus*), 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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