Sequence-Selective Binding of Amiloride to DNA†

Christian Bailly,‡ Alan W. Cuthbert, Dean Gentle, Michael R. Knowles,§ and Michael J. Waring*

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ, U.K.

Received September 2, 1992; Revised Manuscript Received December 29, 1992

ABSTRACT: Nuclease footprinting techniques have been employed to investigate the interaction between the diuretic drug amiloride, a sodium channel blocker with potential therapeutic use in the treatment of cystic fibrosis, and three DNA fragments of defined sequence. Using either DNAse I or micrococcal nuclease as probes, an unusual pattern of sequence-selective recognition of DNA has been detected. Amiloride binds selectively to sites rich in adenine and thymine residues, frequently with an apparent preference for 5′-TpX-3′ steps, and discriminates strongly against GC-rich sequences which are sometimes cut more readily in the presence of the drug compared to the control. A detailed comparison with the actions of known selective DNA-binding antibiotics and drugs reveals a unique pattern of binding sites, different from those of typical intercalators on the one hand and those of minor groove-binders on the other. Amiloride is believed to adopt a pH-dependent tricyclic hydrogen-bonded conformation in solution which allows it to intercalate into DNA; consistent with this belief, we find that the footprinting pattern largely disappears at pH values above the pK_a. Preliminary studies with three amiloride analogues have indicated the importance of two functional groups in the recognition of DNA. The possible relevance of selective DNA binding to activity in vivo is considered.

Amiloride (Figure 1) was introduced into medical practice as a potassium-sparing diuretic (Glitzer & Staelman, 1966). In model epithelial systems it was shown to block sodium channels (Cuthbert et al., 1979), and in competition studies with sodium ions it was shown to have an affinity approaching 10^8 M^{-1} for the epithelial sodium channel (Cuthbert & Shum, 1974). At higher concentrations, amiloride was found to block the Na^+/H^+ (Aronson, 1985) and the Na^+/Ca^{2+} exchangers (Schellenberg et al., 1985). At yet higher concentrations it can inhibit transporting enzymes such as the Na–K-dependent adenosine triphosphatase (Saltoff & Mandel, 1983).

More recently amiloride has been shown to have effects on systems not known to involve sodium ions or sodium-binding processes. In particular, it has been found to inhibit mitogen-induced DNA synthesis (L'Allemain et al., 1984) and RNA transcription (Besterman et al., 1984). The evidence accumulated to date indicates that amiloride binds to DNA by a process of intercalation in such a manner as to inhibit DNA topoisomerase II (Besterman et al., 1987). Thus, whereas sodium channels must continue to be regarded as the principal cellular targets of this drug, it seems to have an important, if subsidiary, target at the level of the gene. The aim of the present work was to determine whether the interaction with DNA involves a sequence-specific recognition process. Accordingly, we chose to employ the technique of footprinting to locate the binding sites for amiloride on three DNA restriction fragments of defined sequence.

Previous reports from this laboratory have addressed the properties of a large variety of drugs that bind to DNA in a sequence-selective manner [see, for examples: Low et al. (1984a,b); Fox and Waring (1986, 1987a,b); Chaires et al. (1987); Portugal and Waring (1987, 1988); McLean et al. (1989); Bailly et al. (1990, 1992)]. Extension of these studies to amiloride has not only fulfilled the objective of detecting sequence-specific binding but has also led to the discovery of a ligand whose recognition properties are very different from those of such classical intercalators as ethidium, daunomycin, or ellipticine.

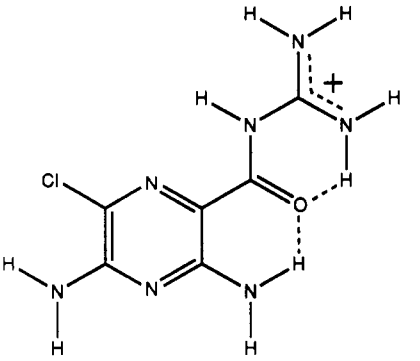

FIGURE 1: Structure of amiloride [3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazinecarboxamide]. The hydrogen-bonded cyclic form is shown.

Doubtless the unusual behavior of amiloride is related to its peculiar chemical structure: it is a pyrazine substituted with various functional groups (Figure 1). Although it contains only one bona fide aromatic ring, it can adopt a tricyclic form due to hydrogen-bonding interactions between the acylguanidino group at position 2 and the amino group at position 3 (Smith et al., 1979). The resulting planar structure, stable in solution, bears a plausible resemblance to the chromophores of classical intercalators and is postulated to be responsible for the intercalative DNA-binding properties of the drug (Besterman et al., 1987).

It is likely that such a conformation will only occur when the drug is in the protonated form, i.e., predominantly at pH < pK_a. Amiloride, because of its guanidino moiety, is a weak base, pK_a = 8.7 (Cuthbert, 1976); therefore, in the physiological pH range, amiloride exists primarily as a monovalent cation. Due to its ionization, it is thought that amiloride binds

† This work was supported by grants from the Cancer Research Campaign, the Medical Research Council, and the Royal Society to M.J.W. and from the Cystic Fibrosis Trust to A.W.C.

* Author to whom correspondence should be addressed.

‡ Present address: INSERM Unité 16, Place de Verdun, 59045, Lille, France.

§ Present address: Dept. of Medicine, Division of Pulmonary Diseases, University of North Carolina, Chapel Hill, NC.

0006-2960/93/0432-2514$04.00/0

electrostatically to a phosphate on the DNA backbone, providing an anchorage for intercalation of the molecule. Bearing this in mind, we have performed DNAse I footprinting experiments at several values of pH. Our results clearly demonstrate that the binding of amiloride to DNA is strongly influenced by pH, with the strongest binding (i.e., the clearest footprints) occurring only at pH < pK_a.

The present results are compared to those previously reported with various well-known DNA-intercalating drugs, and the implications of these findings for the mechanism of action of amiloride are considered.

MATERIALS AND METHODS

Drugs and Enzymes. Amiloride was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions (4 mM) were prepared in different buffers according to the desired pH. DNAse I was purchased from Sigma and stored as a 7200 units/mL solution in 150 mM NaCl, 1 mM $MgCl_2$ at −20 °C. Micrococcal nuclease was purchased from Boehringer (Mannheim) and stored as a 2500 units/mL solution in 50 mM Tris-HCl, pH 7.6, containing 2 mM $CaCl_2$. These stock solutions were diluted to working concentrations immediately before use.

Buffers. For experiments using DNAse I, stock solutions of amiloride were diluted appropriately, and the reaction mixtures were set up in 10 mM Tris-HCl, 10 mM NaCl buffer, pH 7.2, in 20 mM Tris-HCl, pH 8.0, or in 20 mM sodium borate, pH 10.5, according to the desired pH of the solution. The drug was dissolved in 50 mM Tris, 2 mM $CaCl_2$, pH 7.5, for experiments with micrococcal nuclease. Under all conditions, the solubility of the drug was well above that required to prepare 4 mM stock solutions, and no evidence was found for precipitation with other components of reaction mixtures up to the highest concentration of amiloride employed. Checks were made to verify that no detectable interaction occurred with the nucleases themselves. The digestion buffer used to dilute DNAse I to working concentrations contained 20 mM NaCl, 2 mM $MgCl_2$, and 2 mM $MnCl_2$. Inclusion of Mn^{2+} as well as Mg^{2+} in the buffer speeds up the cutting rate 50-fold without changing the DNA control digestion pattern.

DNA Fragments. Plasmid pKMΔ-98 was amplified in *Escherichia coli* by culture, isolated, and purified by cesium chloride ethidium density gradient centrifugation. The 160-base-pair *tyr* T DNA fragment containing the tyrosine tRNA promoter was cut out of the plasmid pKMΔ-98 and labeled according to previously described procedures (Drew & Travers, 1984). Incubation with the Klenow fragment of DNA polymerase I (Boehringer) and [α-^{32}P]dCTP led to selective labeling of the 3′-end of the top strand (Watson strand), whereas incubation with [α-^{32}P]dATP led to selective labeling of the 3′-end of the bottom strand (Crick strand).

The plasmid pBS (Stratagene, La Jolla, CA) was digested to completion with *Pvu*II followed by either *Ava*I or *Eco*RI restriction enzymes, giving products (the 117-bp fragment *Pvu*II/*Eco*RI and the 253-bp fragment *Pvu*II/*Ava*I) suitable for 3′-end labeling. The enzymes were heat-denatured, and two ethanol precipitations were carried out before 3′-end labeling with [α-^{32}P]dCTP or [α-^{32}P]dATP (60 μCi) using the Klenow fragment of DNA polymerase I (20 units) under standard conditions. The DNA fragments were then separated on a 6.5% preparative polyacrylamide gel by electrophoresis for 2 h at 200 V in TBE buffer (8.9 mM Tris base, 8.9 mM boric acid, 2.5 mM Na_2EDTA, pH 8.3). The 117-bp and 253-bp bands were identified by autoradiography, excised, and isolated by elution in 500 mM ammonium acetate, 10 mM magnesium acetate buffer.

DNAse I and Micrococcal Nuclease Footprinting, Gel Electrophoresis, Autoradiography, Densitometry, and Data Processing. The procedure for footprinting experiments was exactly as previously described (Low et al., 1984a; Drew & Travers, 1984; Fox & Waring, 1987a,b). Briefly, the cleavage reaction was initiated by adding 2 μL of nuclease solution (at a concentration adjusted to optimize the cutting rate) to the drug–DNA solution. The reaction was allowed to proceed for 1, 5, and sometimes 30 min before it was quenched by addition of 3 μL of 80% formamide containing 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The cleavage products from the nuclease reactions were denatured at 90 °C for 3 min before electrophoresis on a denaturing polyacrylamide sequencing gel (0.3 mm thick polyacrylamide gel containing 8 M urea and Tris–borate–EDTA buffer, pH 8.3). The percentage of polyacrylamide in the gel ranged from 7 to 10%, depending on the length of the DNA fragment and the region for which high resolution was desired. After 2 h of electrophoresis at 1600 V, the gel was soaked in 10% acetic acid for 15 min, transferred to Whatman 3MM paper, dried under vacuum at 80 °C, and subjected to autoradiography (Kodak, X-OMAT AR) at −70 °C with an intensifying screen.

Autoradiographs were scanned using a multichannel computer-operated gel scanner (Smith & Thomas, 1990). Gel profiles were plotted and displayed on a raster graphics screen. The area under each peak was integrated by simple addition of the pixels under the curve as described in detail by Drew and Travers (1984). In comparing different digestion patterns, care was taken to ensure that the extent of digestion was similar and limited to 20–40% of the starting material so as to minimize the incidence of multiple cuts in any one strand. Data are presented in the form ln (f_a) − ln (f_c) representing the differential cleavage at each bond relative to that in the control (f_a is the fractional cleavage at any bond in the presence of the drug, and f_c is the fractional cleavage of the same bond in the control). The results are displayed on a logarithmic scale for the sake of convenience, so that positive values indicate enhanced cleavage whereas negative values indicate blockage.

RESULTS

To detect specific DNA-binding processes, two complementary enzymatic footprinting methods were used employing either DNAse I or micrococcal nuclease as cleaving agents. In these assays, a labeled DNA fragment is incubated with the drug under investigation, and the complex is submitted to the action of an endonuclease which can cleave each sugar–phosphate bond of the DNA. The products of degradation are then resolved by denaturing gel electrophoresis.

DNAase I Footprinting Experiments. In the first series of experiments, the test DNA was an *Eco*RI–*Ava*I restriction fragment of the plasmid pKMΔ-98 expressed in *E. coli*. This 160-bp fragment, usually referred to as the *tyr* T fragment, directs the synthesis of a major species of tyrosine tRNA (Travers et al., 1983). Figure 2 shows the electrophoresis pattern generated by resolution of the partial DNAse I cleavage products from the *tyr* T DNA in the presence or absence of amiloride. The DNAse I cleavage reaction itself shows some sequence specificity, but this specificity is sufficiently weak to permit cleavage between virtually all of the DNA nucleotides, so that inhibition at any nucleotide along the lattice can be detected. In each case, the digestion procedure was repeated several times in the presence of varying concentrations of amiloride in order to catch intermediate states of drug-induced protection from cleavage.

There is a more or less continuous ladder of DNA fragments in the control lanes resulting from incubation of *tyr* T DNA

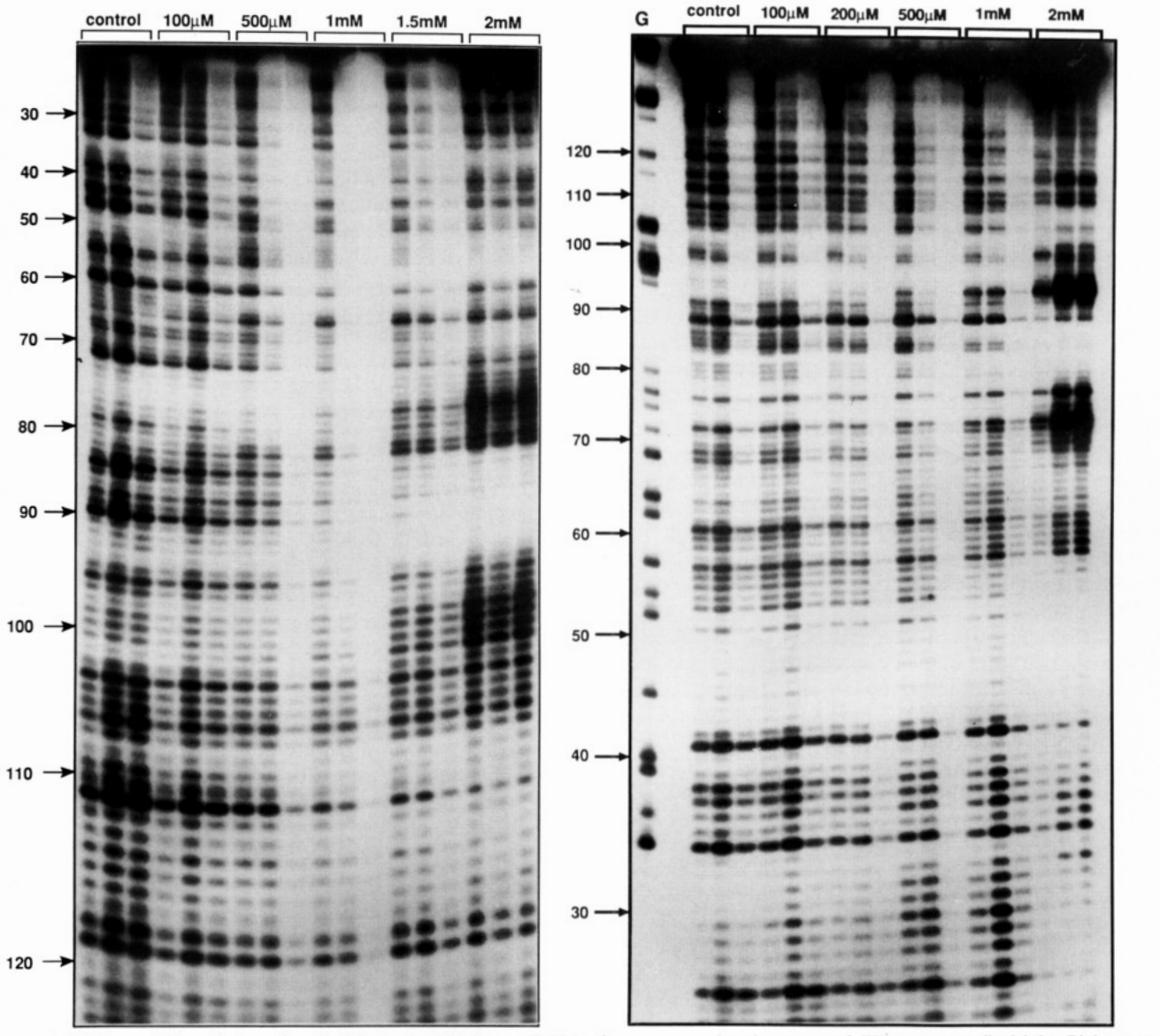

FIGURE 2: DNAase I footprinting of amiloride on the 160-bp *tyr* T DNA fragment: (left, A) top strand (Watson strand); (right, B) bottom strand (Crick strand). The concentration of amiloride tested is shown at the top of the appropriate gel lane. Each set of three lanes corresponds to digestion by the enzyme for 1, 5, and 30 min at pH 7.0. The track labeled "G" represents a dimethyl sulfate-piperidine marker specific for guanine. Numbers at the left side of the gel locate bands with reference to the sequence shown on the corresponding differential cleavage plot in Figure 3.

in the presence of DNAse I. The ladder arises from nicking of the *tyr* T DNA so as to produce fragments differing in length by single nucleotide units. It is apparent in the control lanes of Figure 2 that the intensities of the bands, and hence the relative amounts of nicking at each nucleotide position, vary along the DNA sequence. However, the stopping of the DNAse I reaction at different stages of completion (after 1, 5, and 30 min of incubation) ensures that at least one lane from each incubated sample contains an appropriate distribution of fragment lengths, even when the cutting reaction is substantially inhibited.

In the presence of amiloride, it is obvious that the cleavage pattern differs from that seen in the untreated control samples, suggesting that amiloride is indeed capable of interacting with DNA in a sequence-selective fashion. At relatively low amiloride concentrations (100–200 μM), the cleavage pattern is only weakly altered compared to the control. With increasing concentrations, the involvement of a specific binding process becomes unambiguous as judged from the blockage of the enzyme activity at defined internucleotide bonds. The clearest footprints can be seen with amiloride concentrations around 1.5 mM.

In order to examine the amiloride-induced changes in nuclease cleavage on a more quantitative basis, the average ratio of fractional cleavage (calculated as described in the Materials and Methods section) in the presence of drug to the fractional cleavage in the drug-free control was calculated for each phosphodiester linkage and plotted as in Figure 3A. In this type of representation, phosphodiester bonds protected from attack by DNAse I (indicating steric blockage of the enzyme by amiloride) appear with negative values. Conversely, phosphodiester bonds which are cut better in the presence of amiloride appear as positive values. On the whole, regions of protection or enhanced cleavage appear in roughly the same positions on either strand, although in some places they appear to be staggered, with a 1–2-bp shift in the 3′-direction needed for perfect alignment. This is due to the fact that DNAse I cuts by binding to phosphate groups lying opposite each other across the minor groove of the helix (Fox & Waring, 1987a).

Examination of the gels and the corresponding plots (Figures 2 and 3) shows that common to both strands of the *tyr* T DNA duplex is inhibition of cleavage by amiloride at phosphodiester bonds between bases 21–27, 50–56, 81–91, and 108–114. The

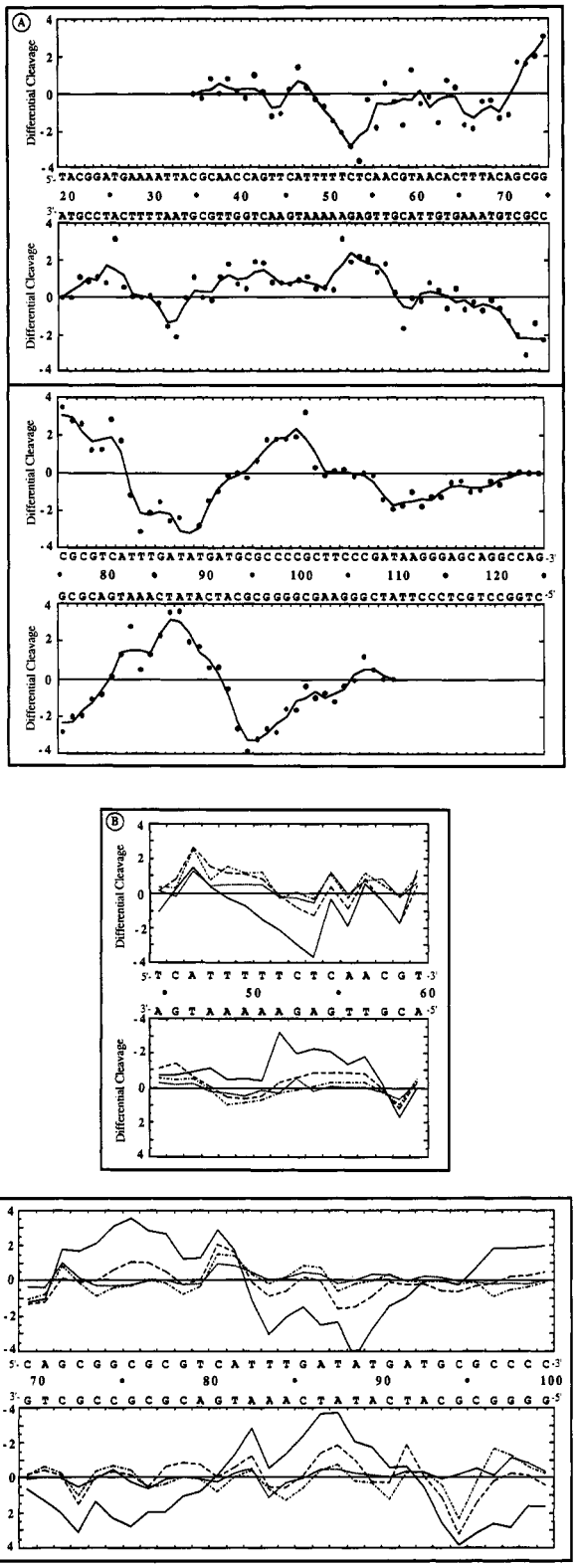

FIGURE 3: (A) Plot of relative (normalized) sensitivity to cleavage by DNAse I in the presence of 2 mM amiloride bound to the *tyr* T DNA at pH 7.0. Actual data points are drawn as black dots representing the altered probability of cleavage at each bond. The smooth line is a three-bond running average calculated by averaging the value at any bond with those of its two nearest neighbors on the same strand. Vertical scales are in units of $\ln(f_a) - \ln(f_c)$, where f_a is the fractional cleavage at any bond in the presence of the drug and f_c is the fractional cleavage of the same bond in the control. Positive values indicate enhancement and negative values blockage. Deviation of experimental points toward the lettered sequence corresponds to a drug protected site, and deviation away represents enhanced cleavage. (B) and (C) Close-up views of the differential cleavage plot in A between nucleotides 70 and 100 and 45 and 60, respectively, showing the dependence upon concentration of amiloride: —, 2 mM; - - -, 1 mM; - · - ·, 500 μM; ···, 100 μM.

two sites around positions 25 and 110 can be unequivocally identified on both strands of the DNA, but because the densitometric analysis is limited to reasonably well-resolved regions of gels where accurate and unambiguous assignment of the chemical identities of the bands can be made, these two sites are represented on only one strand of the DNA in Figure 3. Concentrations of amiloride from 2 to 4 mM produce exactly the same pattern of effects (data not shown).

Almost total absence of cleavage around position 86, signifying tight and specific binding, is evident with 2 mM amiloride. The binding site located around nucleotide position 52 seems to be marginally less favored than the one situated around position 86. But in fact, this site lies in an area of the DNA fragment which is intrinsically more refractory to the endonucleolytic activity of the enzyme and is thus less clearly revealed by the densitometric analysis in Figure 3. It is likely that the two binding sites around positions 52 and 86, corresponding to the sequences 5′-TTTCTCAAC and 5′-TTTGATATG, respectively, are more or less equally and specifically recognized by amiloride. The validity of these sites will be confirmed by the use of micrococcal nuclease (vide infra). These two DNA sequences represent the tightest binding sites of amiloride on *tyr* T DNA.

All four amiloride binding sites (around positions 25, 53, 86, and 111) detected on this DNA fragment are situated in, or adjacent to, AT-rich regions of the DNA. It is difficult from these data to define exactly where the amiloride is bound in each case, since the protected regions are relatively large. But we noticed that the dinucleotides TpA and TpC are practically always associated with the central part of the footprints and might prima facie be considered as the preferred binding site of the drug. However, these two dinucleotide steps are not always protected; for example, the sequence 3′-TAAT around position 32 of the Crick strand lies in a region of enhanced cleavage. Evidently some other factor has to be considered. We note that the protection at a TpA or TpC step seems to be favored when such a step is juxtaposed to a short run of A or T (see the binding sites around positions 25, 53, and 86).

A more cogent explanation as to what is happening in the AT domains is suggested by a study of the concentration dependence of the DNAse I digestion patterns in the presence of various amounts of amiloride (Figure 3B,C). With 2 mM amiloride, the protected sites are broad and do not allow an accurate localization of the binding sites, since the footprints appear far larger than expected from the size of the drug. At lower amiloride concentrations the extent of protection is weaker, but reveals (for the binding site located between positions 81 and 91) that the protection is chiefly centered around positions 84 and 88, i.e., at the dinucleotide steps TpT and TpA, respectively (Figure 3B).

Similar analysis of the concentration dependence of protection at the binding site between positions 49 and 57 reveals (Figure 3C) that raising the drug concentration from 100 μM to 2 mM favors protection at the TpC phosphodiester bond 52 relative to the CpT neighboring bond (most obvious on the bottom strand). Careful analysis of the effect of various concentrations of drug at defined positions leads us to believe that the binding of amiloride is selective for any of the 5′-TpX-3′ steps (complementary to 3′-ApX-5′).

As well as protecting from DNAse I cleavage in the regions already discussed, amiloride induces enhanced cutting at certain bonds relative to that in the control. The regions where cleavage by the enzyme is enhanced are mostly rich in G+C residues, as around positions 75 and 97 of the *tyr* T DNA. Most of the time the enhanced cutting occurs adjacent to sites

where amiloride is bound. It may thus be postulated that these enhancements are a consequence of the protection at neighboring sites. Indeed, inspection of the concentration dependence of binding (Figure 3B,C) reveals that the magnitude of the enhancement chiefly centered at the dinucleotide step 5′-CpG (positions 73, 76, 78, and 95 in Figure 3B) is proportional to the intensity of protection at the flanked binding sites.

Most likely enhanced cleavage occurs as a result of conformational changes induced in the DNA by binding of amiloride to neighboring sites, as discussed previously with other sequence-selective ligands (Low et al., 1984a), although the mass-action effect described by Goodisman and Dabrowiak (1992) may well play an important part. In the mass-action effect the removal of potential nuclease cutting sites by drug binding increases the effective concentration of the remaining sites and, hence, the probability of their being cut. At all events, it is clear that amiloride binding discriminates strongly against GC sequences. This finding will be discussed in detail in the next section, but it can already be stated that amiloride behaves very differently from classical intercalators like actinomycin or ellipticine which are GC selective (Fox & Waring, 1984; Bailly et al., 1990).

Further footprinting experiments were conducted with two DNA fragments obtained from the plasmid pBS which contain quite different arrangements of AT and GC clusters. DNAse I cleavage of the 253-bp and 117-bp DNA fragments again affords a nonuniform DNA cleavage pattern, as observed in sequencing gels (see the 253-bp fragment in Figure 4, for example). All of the internucleotide bonds can be cleaved but with varying intensity. Treatment of the DNAs with amiloride leads to changed patterns, especially with 2 mM amiloride, that are due to the inhibition of DNAse I cleavage at sites where the drug is bound superimposed upon enhanced cleavage between binding sites. However, the modified cleavage patterns with these DNA fragments are more evident from the strongly enhanced cleavage by DNAse I at positions where the drug does not bind than from true footprints, i.e., clear blockage of the enzyme cutting at particular sites. Evidently these DNAs do not offer prime sequences specifically recognized by amiloride, unlike *tyr* T DNA, and the dinucleotide 5′-TpA is notably underrepresented in these two DNA fragments. Thus, the few sites where DNAse I cleavage is diminished in the presence of the drug are probably best considered as secondary binding sites. The existence of a graded intensity of enhancement or protection with increasing amiloride concentration is common to the pBS fragment and the *tyr* T DNA: it suggests that the observed patterns result predominantly from sequence-specific binding of the drug to sites of differing affinity rather than from drug-induced DNA conformational changes.

Figure 5 shows differential cleavage plots for the two pBS DNA fragments measured in the presence of 2 mM amiloride. With the 117-mer (lower panel), four regions centered around positions 48, 71, 88, and 97 are protected from cleavage. A similar picture emerges with the 253-mer, where some sequences show an attenuated, though not totally blocked, cleavage by DNAse I. Sites which can be identified on both strands (data not shown) occur around positions 46, 56, 102, and 111. In every case the protection is relatively weak and, as with the *tyr* T DNA, often lies close to the dinucleotide steps TpA or TpC. However, a clear preference for these dinucleotides cannot be evidenced. For example, with the 253-mer, one binding site is revealed between positions 98 and 107 corresponding to the sequence 3′-ACACACTTT. Thus the dinucleotide 3′-ApC (complementary to 5′-TpG)

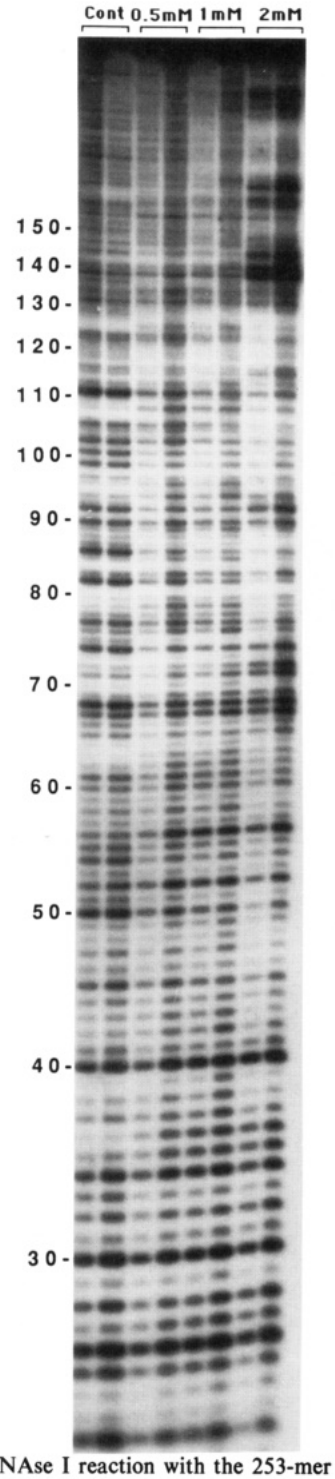

FIGURE 4: DNAse I reaction with the 253-mer fragment *Pvu*II–*Ava*I of the plasmid pBS in the presence of different concentrations of amiloride (0.5, 1, and 2 mM) at pH 7.0. The tracks labeled Cont contained no drug. Numbers at the left side of the gel refer to the sequence shown on the corresponding differential cleavage plot in Figure 5. Other details as for Figure 2.

may also provide a binding site for amiloride, but is less favored than the dinucleotides 5′-TpA or 5′-TpC. There is, in fact, what looks like a nice isolated site nearby surrounding the 5′-TpA at position 112. It must be noted that both of these sites are juxtaposed to a run of T and A nucleotides, as mentioned with the *tyr* T fragment. However, homopolymeric sequences do not generally provide a binding site for amiloride since these sequences frequently correspond to sites of enhanced DNAse I cleavage. This is particularly obvious for runs of G and C and for GC-rich sequences where the strongest enhancements have been observed. Dramatically increased

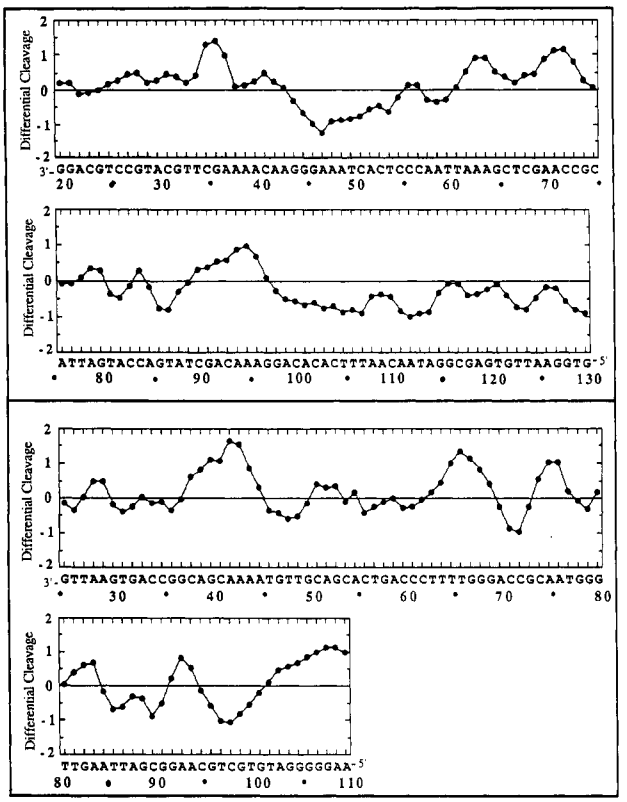

FIGURE 5: Differential cleavage plots for the 253-bp DNA fragment (upper panel) and the 117-bp DNA fragment (lower panel) of the plasmid pBS by DNAse I in the presence of 2 mM amiloride at pH 7.0. Data for only one strand are shown. Presentation as described in the legend to Figure 3.

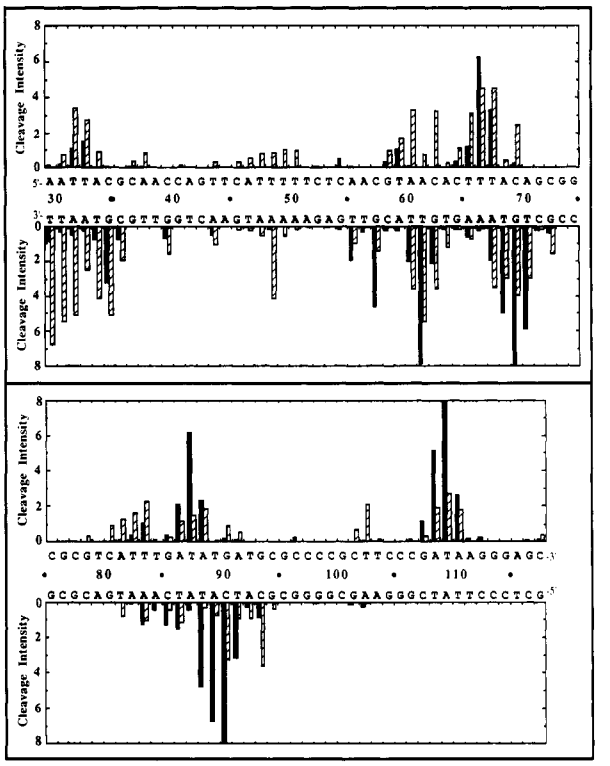

FIGURE 6: Micrococcal nuclease cutting map for *tyr* T DNA in the absence (filled bars) and presence (hatched bars) of 2 mM amiloride. The heights of the bars correspond to the relative probability of cutting at each bond, expressed on a linear scale. Data extend over positions 30–118 (top strand) and 30–105 (bottom strand).

rates of DNAse I cleavage can be observed around positions 143 and 168 of the 253-mer. Accurate densitometric analysis cannot be performed at these regions on the gel presented in Figure 4. But from other gels where the electrophoresis was extended so as to resolve the closely spaced bands corresponding to longer fragments, we were able to show that these regions of enhanced cleavage correspond to very G+C-rich sequences extending from positions 139 to 146 (3′-GCTCGGCC) and 163 to 174 (3′-CGGACCCCACGG).

In summary, the results from DNAse I footprinting experiments performed with all three DNA fragments correlate well. It appears clearly that amiloride prefers to bind to AT rather than GC sequences. For this reason, we decided to investigate further the nature of the amiloride binding sites by the use of micrococcal nuclease. This enzyme is an AT-selective cutting probe (Fox & Waring, 1987b). Naturally, *tyr* T DNA which contains the tightest amiloride binding sites as judged by DNAse I footprinting was selected as the most suitable substrate.

Micrococcal Nuclease Footprinting. Electrophoresis patterns generated by resolution of the partial micrococcal nuclease (MNase) cleavage products from *tyr* T DNA in the presence of different concentrations of added amiloride were quantitated by microdensitometry and converted to the histogram plots shown in Figure 6. The histograms show the relative cleavage by MNase of drug-treated versus control DNA (Fox & Waring, 1987b).

MNase cuts almost exclusively at pA and pT internucleotide bonds but is also sensitive to the nature of their flanking sequences. Because its cutting pattern on the control DNA differs greatly from that produced by DNAse I, it yields complementary information on the selectivity of drug binding. By inspection of the plots in Figure 6, it is apparent that the internucleotide bonds cleaved in the absence of drug are still measurably cleaved in the amiloride-containing sample. The substantial modifications of the cleavage pattern caused by the presence of 2 mM amiloride arise mainly from the appearance of numerous new cleavage sites rather than from inhibition of preexisting cutting sites. For example, in the absence of drug very little or no cutting is observed in the homopolymeric run of five AT pairs on either strand of the *tyr* T DNA. In the presence of 2 mM amiloride, cleavage at this region (47–52) is definite and even marked (position 49).

With DNAse I, a strong amiloride binding site was detected between positions 82 and 91 with major protection at the 5′-TpA dinucleotide (position 88). MNase confirms this sequence as a preferred amiloride binding site. The intense cutting observed in the absence of the drug at this sequence is very strongly reduced with amiloride (in percentage terms, maximal inhibition occurs directly at the 5′-TpA site). Moreover, regions surrounding this sequence are over-cut by MNase in the presence of amiloride. Similar agreement between DNAse I and MNase can be observed at other positions of the *tyr* T DNA, notably, the AT-rich region located around position 32, which is clearly disfavored by the drug since both enzymes show enhanced cutting at this site. The binding site revealed at position 53 by DNAse I cannot be identified with MNase because even in the absence of drug this sequence is refractory to cutting by the enzyme.

Thus, on the whole MNase footprinting reveals that the apparent binding sites are real and not merely an artifact of using DNAse I. It confirms that the diuretic drug amiloride is a sequence-selective, DNA-binding ligand.

pH-Dependent Binding to DNA. Figure 7 shows autoradiographs of gels from DNAse I digestion of the two strands of *tyr* T DNA in the presence and absence of amiloride at different values of pH. The two selected pH values lie on either side of the pK_a of the drug (pK_a = 8.7). At pH 8.0, the regions of protection from DNAse I cleavage described

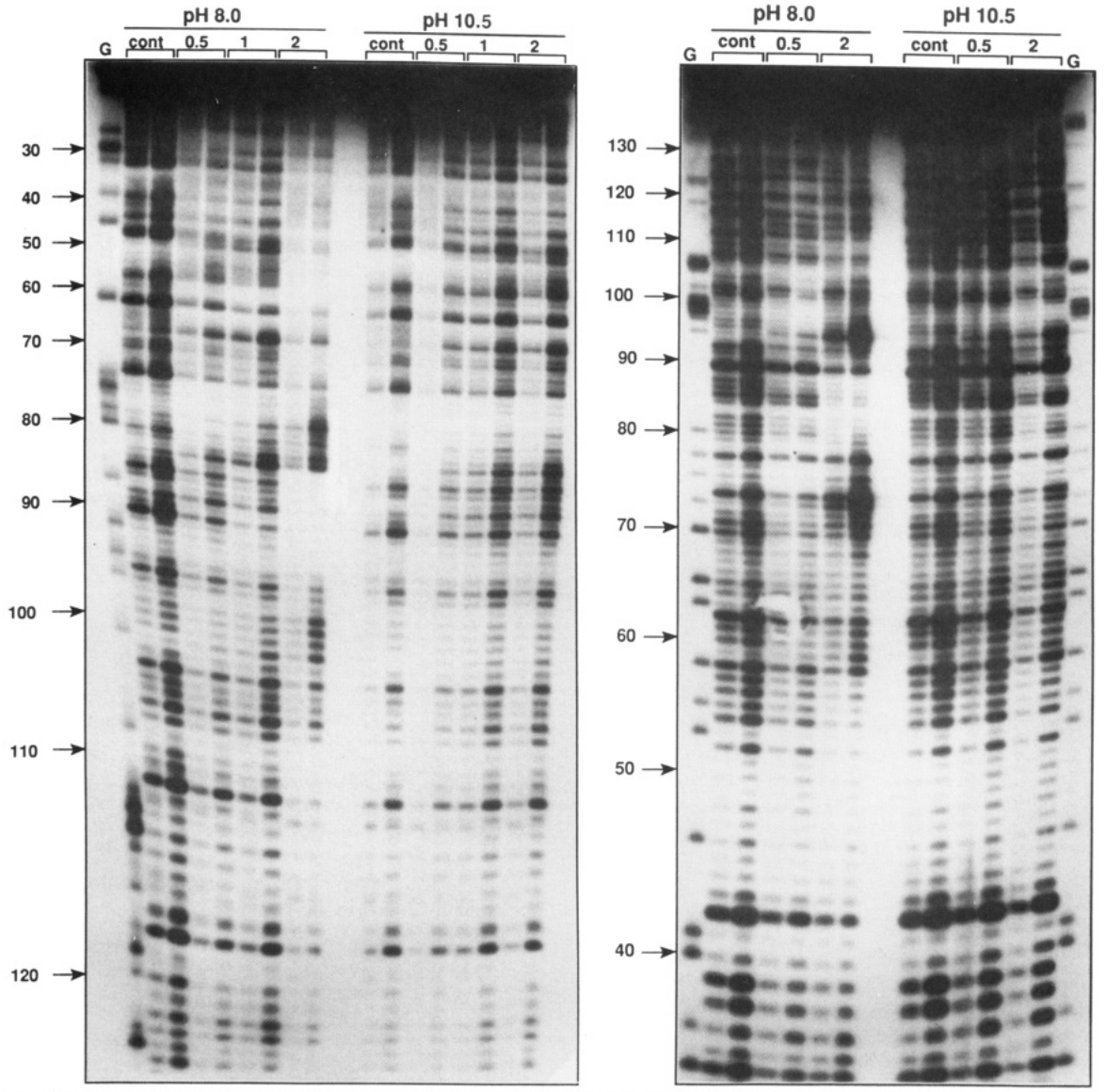

FIGURE 7: Effect of pH on the DNAse I reaction with (left, A) the Watson strand and (right, B) the Crick strand of the *tyr* T DNA fragment in the presence of different concentrations of amiloride (0.5, 1, and 2 mM). The left-hand panel shows experiments carried out at pH 8.0, whereas the right-hand panel shows experiments carried out at pH 10.5. Other details as for Figure 2.

above at pH 7.0 are still clearly visible, with the most prominent binding around position 86. The positions of the protected and enhanced regions remain constant at pH values ranging from 4.0 to 8.0. Moreover, the magnitude of the protection and enhancement varies little, if at all, over the pH range between 4.0 and 7.5 (data not shown) and is only slightly reduced at pH 8.0.

Similar DNAse I footprinting experiments performed at pH 10.5 reveal that the effect of amiloride is substantially reduced. It should be noted that the enzyme activity is itself minimally affected by varying the pH. Only slightly more DNAse I is required at pH 10.5 (0.042 unit/mL) compared to the concentration used at pH 7.0 (0.036 unit/mL). Thus when the drug is in the basic form, its DNA-binding properties are not completely abolished, but are greatly diminished. A few weakly protected DNA sequences can still be observed (Figure 8), but their magnitudes are in no way comparable to the clear footprints detected at pH < pK_a. The DNAse I cleavage pattern observed in the presence of 2 mM amiloride at pH 10.5 is approximately comparable to the one obtained with 500 μM amiloride at pH 8.0 (compare the relevant tracks in Figure 7, especially between bands 75 and 90). We estimate that the binding of amiloride to DNA, already weakened at pH 8.0, is further reduced 4–5-fold at pH > pK_a.

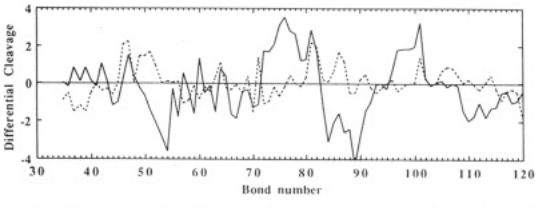

FIGURE 8: Comparative differential cleavage plot for amiloride bound to the Watson strand of the *tyr* T fragment at pH 8.0 (—) and 10.5 (- - -). The form of the plot is described in the legend to Figure 3. The sequence has been replaced by the corresponding bond number and the abscissa is compressed relative to that of Figure 3A.

DISCUSSION

The results presented here provide the first experimental evidence that the diuretic drug amiloride is capable of recognizing specific DNA sequences. The progressive change in the footprinting pattern seen with amiloride over such a wide range of drug concentrations argues in favor of the involvement of sequence-specific binding to different sites which are occupied according to their relative affinities rather than drug-induced changes in DNA conformation. It is unlikely that amiloride induces defined sequences of DNA molecules to adopt a conformation which is resistant to DNAse

I without binding to such sequences. Results from three different DNAs of varied composition and experiments using two independent enzymatic probes are mutually consistent and point to the same sequence selectivity.

Sequence Specificity and Mode of Binding. We find that amiloride strongly discriminates against GC-rich sequences and manifests a general preference for AT-rich sites in DNA. This behavior immediately distinguishes amiloride from most other intercalators. Its apparent AT preference contrasts sharply with the preference of typical intercalators which are more often GC selective (Müller & Crothers, 1975). Although intercalative binding and AT selectivity are not incompatible (Wilson et al., 1985), the present results prompt the question of whether or not amiloride is truly an intercalating agent. The possibility that amiloride may not intercalate properly can be ruled out for the following reasons: (i) The previous study by Besterman et al. (1987) includes helix unwinding and extension data which leave little room for doubt that amiloride affects the properties of DNA in the fashion typical of acknowledged intercalators (Neidle & Waring, 1983). (ii) Electric linear dichroism measurements on amiloride–DNA complexes give consistently negative dichroism signals, which show that the plane of the drug molecule is oriented parallel to that of the DNA bases, a geometry which is not consistent with groove-binding (C. Bailly and C. Houssier, unpublished observations). (iii) It frequently appears that the protection against nuclease cleavage is confined to dinucleotide steps. This supports an intercalative mode of binding rather than a groove-binding process, which would generally span a longer site. (iv) The limited structure–activity relations available (see below) are consistent with the need for a planar, tricyclic conformation. (v) The concentration dependence of footprinting speaks for a smooth progression from weak protection at a small, discrete number of sites (observable at concentrations below 100 μM) to strong protection at the same sites with a larger footprint at concentrations up to 40-fold higher. This behavior would be unlikely for any mixed binding mode. For all of these reasons it would be perverse to argue that the footprinting results from sequence-specific external binding and that the intercalated drug does not generate the footprints observed. However, there is no denying that the binding constant must lie at the weaker end of the range encountered with known DNA-binding drugs, probably somewhere in the range 10^3–10^4 M^{-1} judging by the intensity of the footprints. Besterman et al. (1987) measured an apparent K_d of 1.3 mM for binding of amiloride to isolated cell nuclei and noted some ionic strength dependence of the interaction with purified DNA, consistent with a binding constant in this range.

Although amiloride does not display any absolute sequence-binding requirements, it is noteworthy that regions protected from enzyme attack are always rich in A and T residues. However, runs of contiguous A or T nucleotides alone do not constitute good binding sites since they often appear in regions of enhanced cleavage. The presence of such zones of DNAse I hypersensitivity appears as a common feature of amiloride and other sequence-selective DNA-binding ligands (Low et al., 1984a; Fox & Waring, 1984, 1986; Portugal & Waring, 1987).

An important conclusion to be drawn from the concentration dependence experiments is that no particular 5′-TpX dinucleotide step constitutes a uniquely preferred binding site. Many of the protected regions in *tyr* T DNA contain the dinucleotide step 5′-TpA (68, 88, 110), 5′-TpC (24, 27, 45, 52, 86, 91, 108), and 5′-TpG (26, 41, 46, 55, 81, 90), but not all such sequences are protected in any of the DNA fragments we have studied here. The magnitude of DNAse I inhibition at these different dinucleotide steps varies with the nature of the dinucleotide. These variations seem to indicate that the preferred amiloride intercalation sites are, in order of decreasing effectiveness, TpA > TpC > TpG. But very likely this apparent order of selectivity only relates to how easily the adjacent base pairs can be disturbed to accommodate the intercalator. The dinucleotide TpA, for example, unstacks more easily than TpG (Ornstein et al., 1978), which would explain why amiloride binds more tightly to the former than to the latter.

Comparison with Footprinting Data for Other DNA-Binding Drugs. In Figure 9, the binding sites found here for amiloride are compared with those previously reported on the *tyr* T DNA fragment for several other DNA-binding ligands. It is obvious that the protection pattern found for amiloride differs from that produced by the intercalating agents ellipticine (Bailly et al., 1990), daunomycin (Chaires et al., 1987), and ethidium (Fox & Waring, 1987a), each of which exhibits a more or less pronounced GC selectivity. The observed specificity of amiloride most closely resembles the pattern reported for nonintercalating compounds such as netropsin, distamycin, and berenil. Indeed, amiloride and these minor groove-binders share a preference for AT-rich sequences of the DNA. But the pattern produced by amiloride is far from identical to that produced by netropsin, which protects practically all of the AT-containing sites; in the main the sequences protected by amiloride are restricted to a subset of the netropsin sites. Among all of the drugs to have been footprinted on the *tyr* T DNA with DNAse I, it appears that it is the synthetic quinoxaline antibiotic analogue TANDEM (Low et al., 1984b) which produces a pattern of protection most similar to that observed with amiloride (Figure 9). Both the bis-intercalator TANDEM and the mono-intercalator amiloride seem to show a preference for the 5′-TpA dinucleotide. However, TANDEM and amiloride are not structurally related, and we are at a loss to explain these seemingly identical DNA-recognition properties.

Possible Structural Basis of Amiloride Sequence Specificity. The previous proposal (Besterman et al., 1987) that amiloride has to be protonated to bind to DNA is supported by our footprinting experiments carried out in different buffers. At pH 10.5, weak binding is still observed but may be attributed to the remaining protonated fraction of the drug. Although protonation is not absolutely necessary to form the hydrogen-bonded cyclic structure, it can be argued that protonation would stabilize it; accordingly the coplanar tricyclic conformation of amiloride may not survive as the drug becomes deprotonated to the free-base form (Cuthbert et al., 1979; Smith et al., 1979). Thus, if amiloride has to be protonated to be biologically active in regard to sodium channel blockade (Cuthbert, 1976), the ionized form is also required for sequence-specific DNA binding of the drug.

However, this necessary condition is not by itself sufficient to allow selective binding. Pertinent to this point, we have footprinted two amiloride analogues, monosubstituted [N^5-propylamiloride] and disubstituted [N^5-isopropyl-N^5-methylamiloride] on the amino group at position 5. Both of these analogues have relative molar activities of less than 1% that of amiloride (Cuthbert & Fanelli, 1978) and did not show sequence-specific binding (data not shown). We may thus postulate that the exocyclic 5-amino substituent which is not involved in maintaining the planar structure is nevertheless directly implicated in the DNA-binding process. It is likely that this potential hydrogen bond donor substituent participates in the recognition of 5′-TpX by stabilizing a pyrazine/pyrimidine stacking interaction and creating non-Watson–

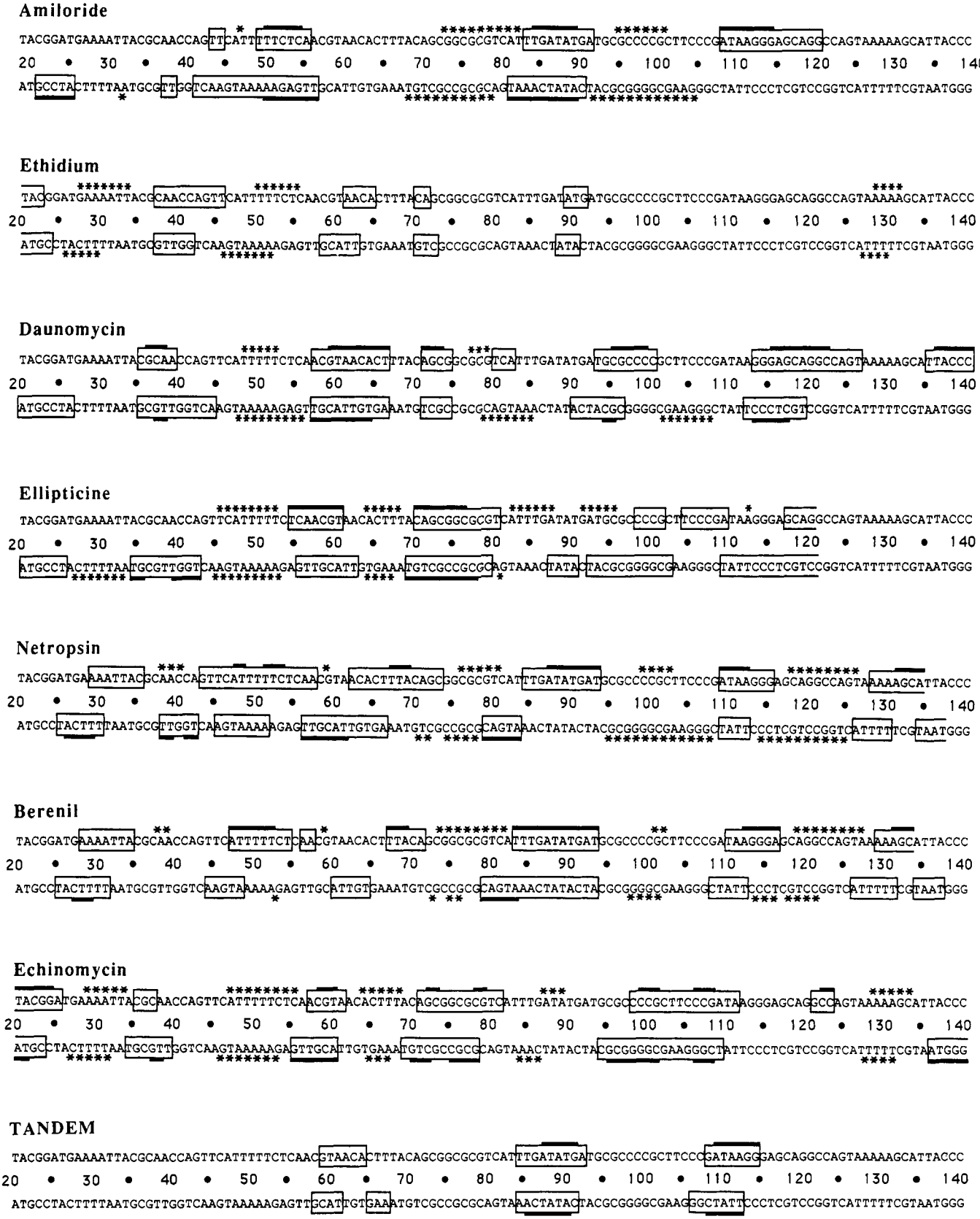

FIGURE 9: Schematic representation comparing the effects of different intercalators (daunomycin, ethidium, ellipticine), bis-intercalators (TANDEM, echinomycin), and minor groove-binders (netropsin, berenil) with those of amiloride on the protection from DNAse I cleavage of the *tyr* T DNA fragment. Protected regions for each drug are indicated by the boxes, with heavy lines indicating major identified drug-binding sites. Asterisks represent sites of drug-induced enhanced cleavage by DNAse I. Compiled from the following: results reported here (amiloride); by Fox and Waring (1987a) for ethidium; Chaires et al., (1987) for daunomycin; Bailly et al. (1990) for ellipticine; Portugal and Waring (1987) for netropsin and berenil; and Low et al. (1984a) for echinomycin and (1984b) for TANDEM.

Crick hydrogen bonds to the thymine carbonyl oxygen at position 2 of the pyrimidine ring or an associated water molecule. Another amiloride analogue, benzamil, has also been tested in our footprinting assays. This compound, which bears a benzyl group on the terminal guanidino nitrogen, has a 10-fold greater affinity for sodium channels than amiloride

(Aceves et al., 1979), but proved unable to bind to DNA in a sequence-selective manner at pH 7.0 (data not shown). Thus the terminal nitrogen atom of the guanidino moiety may also be essential for the DNA-recognition process.

The lack of effect of these analogues might also arise from their increased hydrophobicity (compared to amiloride), which could disfavor the interaction with DNA. A solvent effect might additionally need to be considered: preparation of stock solutions of these compounds requires large amounts of DMSO or DMF, while amiloride can be easily dissolved in aqueous buffer. It would be necessary to test a series of amiloride congeners to determine the structural features of the sequence-specific recognition mechanism. However, it is already plain that any structural change disrupting the planar conformation or masking the hydrogen bond donor groups substantially reduces or inhibits the DNA-binding process.

Finally, we note that the peculiar behavior of amiloride as an AT-selective intercalator lacking the usual chromophoric system of fused aromatic rings is nevertheless consistent with the observations of Wilson and his colleagues (1990a,b) on binding of unfused aromatic cations to DNA. These authors have elegantly demonstrated how a series of closely related compounds containing the same unfused central aromatic ring system and the same charge can interact with DNA by three completely different binding modes. Amiloride adopts the intercalative mode in common with one subset of DNA-binding compounds studied by Wilson et al. (1988, 1989, 1990a,b).

Biological Implications. The present study has a practical aspect since nebulized amiloride has been employed with some success in a recent clinical trial to assist airway function in patients suffering from cystic fibrosis (Knowles et al., 1990). Here the strategy was to exploit the primary action of the drug so as to block sodium channels in airway epithelia and consequently reduce the excessive absorption of fluid which occurs in this disease. The impairment of lung function in patients receiving amiloride was less than in controls, and sputum viscosity was restored to normal, yet the bacterial count was unchanged. In other studies, amiloride was shown to exert a nonspecific bacteriostatic effect on *Staphylococcus aureus* and also to enhance the antibacterial action of tobramycin against *Pseudomonas capacia* (Lisker et al., 1987; George et al., 1988). These findings naturally led to the notion that a direct antimicrobial action of amiloride, perhaps mediated via a selective effect on the bacterial DNA, might play a significant part in the therapeutic effect of the drug. We now find that amiloride–DNA interaction does indeed involve some selectivity, though whether that selectivity has any bearing on its action in vivo remains to be determined. The DNA-binding constant is relatively weak and certainly far weaker than the affinity of amiloride for elements of the sodium channel, but on the other hand it is not necessary for DNA-interactive drugs to bind with high affinity to exert a biological effect because of the enormous effective concentration of their receptor inside cells (Neidle & Waring, 1983; Waring & Ponder, 1992). Moreover, the administration of amiloride to patients in nebulized form is likely to lead to high local concentrations at the surface of airway epithelial cells where even weak DNA binding might represent a serious hazard, especially if administered repeatedly to alleviate chronic disease. In any event, it is important that a fuller understanding of all of the actions of amiloride be gained if it is to be used routinely in the treatment of cystic fibrosis. Amiloride analogues which block sodium channels while exerting a more pronounced antibacterial action, but without endangering the viability of airway epithelial cells, would clearly be advantageous.

ACKNOWLEDGMENT

We thank Dr. C. O'hUigin for advice, and we are especially grateful to Sir Aaron Klug and Dr. J. Smith for providing instructions and access to the gel scanning and computer facilities of the MRC Laboratory of Molecular Biology.

REFERENCES

Aceves, J., Cuthbert, A. W., & Edwardson, J. M. (1979) *J. Physiol. 295*, 477–490.
Aronson, P. S. (1985) *Annu. Rev. Physiol. 47*, 545–560.
Bailly, C., O'hUigin, C., Rivalle, C., Bisagni, E., Hénichart, J. P., & Waring, M. J. (1990) *Nucleic Acids Res. 18*, 6283–6291.
Bailly, C., Denny, W. A., Mellor, L., Bisagni, E., Wakelin, L. P. G., & Waring, M. J. (1992) *Biochemistry 31*, 3514–3524.
Besterman, J. M., Tyrey, S. J., Cragoe, E. J., Jr., & Cuatrecasas, P. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6762–6766.
Besterman, J. M., Elwell, L. P., Blanchard, S. G., & Cory, M. (1987) *J. Biol. Chem. 262*, 13352–13358.
Chaires, J. B., Fox, K. R., Herrera, J. E., Britt, M., & Waring, M. J. (1987) *Biochemistry 26*, 8227–8236.
Cuthbert, A. W. (1976) *Mol. Pharmacol. 12*, 945–957.
Cuthbert, A. W., & Fanelli, G. M. (1978) *Br. J. Pharmacol. 63*, 139–149.
Cuthbert, A. W., Fanelli, G. M., & Scriabine, A. (Eds.) (1979) *Amiloride and epithelial sodium transport*, Urban and Schwarzenberg, Baltimore.
Cuthbert, A. W., & Shum, W. K. (1974) *Naunyn–Schmiedeberg's. Arch. Pharmacol. 281*, 261–269.
Drew, H. R., & Travers, A. A. (1984) *Cell 37*, 491–502.
Fox, K. R., & Waring, M. J. (1984) *Nucleic Acids Res. 12*, 9271–9285.
Fox, K. R., & Waring, M. J. (1986) *Biochemistry 25*, 4349–4356.
Fox, K. R., & Waring, M. J. (1987a) *Nucleic Acids Res. 15*, 491–507.
Fox, K. R., & Waring, M. J. (1987b) *Biochim. Biophys. Acta 909*, 145–155.
George, M., Knowles, M., & Gilligan, P. (1988) *Am. Soc. Microb. A* 167.
Glitzer, M. S., & Stealman, S. L. (1966) *Nature 212*, 191–193.
Goodisman, J., & Dabrowiak, J. C. (1992) *Biochemistry 31*, 1058–1064.
Knowles, M. R., Church, N. L., Waltner, W. E., Yankaskas, J. R., Gilligan, P., King, M., Edwards, L. J., Helms, R. W., & Boucher, R. C. (1990) *New Engl. J. Med. 322*, 1189–1194.
L'Allemain, G., Franchi, A., Cragoe, E., Jr., & Pouysségur, J. (1984) *J. Biol. Chem. 259*, 4313–4319.
Lisker, A., Gilligan, P., Wait, K., & Knowles, M. (1987) *Pediatr. Pulm. 3*, 126.
Low, C. M. L., Drew, H. R., & Waring, M. J. (1984a) *Nucleic Acids Res. 12*, 4865–4879.
Low, C. M. L., Olsen, R. K., & Waring, M. J. (1984b) *FEBS Lett. 176*, 414–420.
McLean, M. J., Seela, F., & Waring, M. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9687–9691.
Müller, W., & Crothers, D. M. (1975) *Eur. J. Biochem. 54*, 267–277.
Neidle, S., & Waring, M. J. (1983) *Molecular Aspects of Anti-Cancer Drug Action*, Macmillan, London.
Ornstein, R. L., Rein, R., Breen, D. L., & MacElroy, R. D. (1978) *Biopolymers 17*, 2341–2360.
Portugal, J., & Waring, M. J. (1987) *Eur. J. Biochem. 167*, 281–289.
Portugal, J., & Waring, M. J. (1988) *Biochim. Biophys. Acta 949*, 158–168.
Saltoff, S. P., & Mandel, L. J. (1983) *Science 220*, 957–959.
Schellenberg, G. D., Anderson, L., Cragoe, E. J., & Swanson, P. D. (1985) *Mol. Pharmacol. 27*, 537–543.
Smith, R. L., Cochran, D. W., Gund, P., & Cragoe, E. R., Jr. (1979) *J. Am. Chem. Soc. 101*, 191–201.

Smith, J. M., & Thomas, D. J. (1990) *CABIOS 6*, 93–99.
Travers, A. A., Lamond, A. I., Mace, H. A. F., & Berman, M. L. (1983) *Cell 35*, 265–273.
Waring, M. J., & Ponder, B. A. J. (1992) *The Search for New Anti-Cancer Drugs*, Kluwer, Dordrecht.
Wilson, W. D., Wang, Y.-H., Kusuma, S., Chandrasekaran, S., Yang, N. C., & Boykin, D. W. (1985) *J. Am. Chem. Soc. 107*, 4989–4995.
Wilson, W. D., Strekowski, L., Tanious, F. A., Watson, R. A., Mokrosz, J. L., Strekowska, A., Webster, G. D., & Neidle, S. (1988) *J. Am. Chem. Soc. 110*, 8292–8299.
Wilson, W. D., Tanious, F. A., Watson, R. A., Barton, H. J., Strekowska, A., Harden, D. B., & Strekowski, L. (1989) *Biochemistry 28*, 1984–1992.
Wilson, W. D., Tanious, F. A., Buczak, H., Venkatraman, M. K., Das, B. P., & Boykin, D. W. (1990a) in *Molecular Basis of Specificity in Nucleic Acid-Drug Interactions* (Pullman, B., & Jortner, J., Eds.) pp 331–353, Kluwer Academic Publishers, Dordrecht.
Wilson, W. D., Tanious, F. A., Barton, H. J., Wydra, R. L., Jones, R. L., Boykin, D. W., & Strekowski, L. (1990b) *Anti-Cancer Drug Des. 5*, 31–42.