Chemical Reagents for Investigating the Major Groove of DNA

A wide array of chemical reagents have been developed over the past 25 years for characterizing DNA structure and its interaction with drugs and proteins. When first introduced, these reagents provided one of a very limited number of approaches for identifying nucleotide sequences that, for example, bound to proteins or formed unusual (non-B helical) structures. More recently, physical methods such as NMR (James, 1995; Addess and Feigon, 1996) and X-ray crystallography (Timsit and Moras, 1992; Sriram and Wang, 1996) have increasingly become viable alternatives since their application has become more routine, providing molecular details of unrivaled resolution. Despite these advances, chemical methods remain highly popular. Their lasting appeal is maintained in part by their low cost and ease of use. Few other techniques have proven as versatile for both defined and complex systems.

Most chemical reagents used to characterize DNA either alkylate or oxidize a particular functional group within one or more of the four standard deoxyribonucleotides. A complementary set of reagents is therefore necessary if an analysis of all polynucleotide domains is desired. Although a single region of DNA such as its major groove may be examined successfully with a limited number of reagents, reliance on a single reagent is not recommended. Data from multiple reagents help to ensure that results truly reflect the state of DNA and are not a function of an unknown or unforeseen idiosyncracy of any one reagent. A number of excellent reviews describe the general specificity and utility of a broad range of reagents, and the most comprehensive of these cover the literature prior to the last decade (Nielsen, 1990; Tullius, 1991; Chow and Barton, 1992; Lilley, 1992). This field has continued to benefit from ongoing development of many additional reagents as illustrated throughout this chapter. The focus of this specific commentary centers on the most commonly used and readily available reagents that react in the major groove: dimethyl sulfate (DMS), diethylpyrocarbonate (DEPC), potassium permanganate ($KMnO_4$), osmium tetroxide (OsO_4), and bromine (Br_2) formed in situ (Ross and Burrows, 1996) from bromide (Br^-) and monoperoxysulfate (HSO_5^-). The characteristics of each reagent will be reviewed individually below, and then a sampling of their broad-ranging applications will be illustrated collectively in the final sections.

GENERAL CONSIDERATIONS FOR DATA ANALYSIS

One of the most attractive features of experiments based on chemical modification is the ability to generate large quantities of data rapidly. However, accurate interpretation of the data is less assured. Assessment of the results requires some familiarity with the basic chemistry of modification, the origins of its specificity, and its potential for ambiguity. With these considerations, strategies may be chosen to distinguish between a limited set of alternative conformations or even explore unknown structures. Ambiguities may still arise when unanticipated variables dominate reaction or when initial modification of a target, or molecules bound to a target, promote one or more secondary reactions. Perhaps the greatest limitation generally affecting chemical modification is set by its ability to report on only the most reactive and not necessarily the most abundant species. These potential problems need not diminish the importance of this approach as long as caution is practiced during experimental design and data interpretation. For example, perturbations caused by secondary reactions can usually be avoided by simply adjusting conditions so that only a small fraction of molecules (<20%) are modified in each analysis. Of course no single experimental technique is ever likely to establish a significant conclusion without corroboration from alternative methods. Chemical modification provides a convenient complement to the more time-consuming procedures based on biological and physical methods.

EXPERIMENTAL PROCEDURES SUPPORTED BY CHEMICAL MODIFICATION

Use of chemical probes typically follows one of three approaches identified as (1) modification protection or "footprinting," (2) interference experiments, and (3) missing contact probing. The first essentially measures the extent of modification along a nucleotide sequence. Uniform reactivity indicates structural homogeneity within DNA, and, conversely, variable reactivity indicates structural heterogeneity at least to the extent of localized in-

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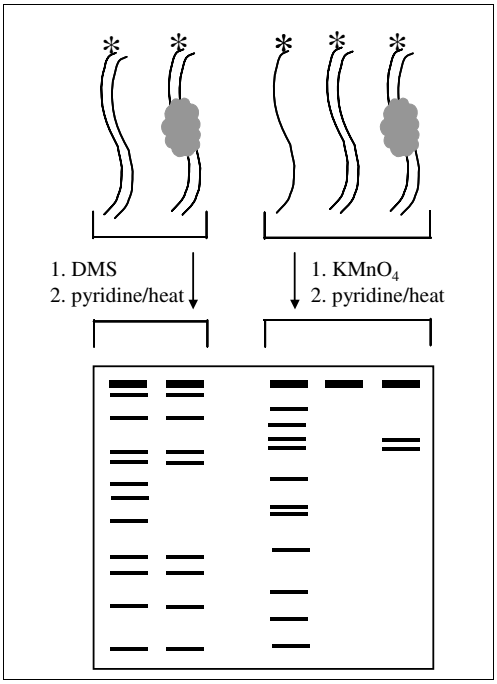

Figure 6.6.1 Protein-dependent suppression and activation of DNA modification by conformation-specific probes.

creases or decreases in steric or electrostatic repulsion. For example, assembly of a protein-DNA complex may be expected to block access to nucleotides in a certain region of a helix and prevent reaction at this site uniquely (Fig. 6.6.1). Likewise, partial unwinding of duplex DNA resulting from a helical junction or bound protein will increase access and concomitant reaction of a selected number of neighboring nucleotides. These sites of interest are identified in both cases by their deviation from the basal level of reactivity established over many nucleotides. Although certain reagents such as hydroxyl radicals induce polynucleotide strand scission directly, the reagents reviewed below modify the pyrimidine and purine bases without causing spontaneous strand scission. The modified bases are instead detected by their diagnostic ability to (1) cause strand scission after subsequent treatment with heat and piperidine (Maxam and Gillbert, 1980; see *CPMB UNIT 7.5*) or (2) terminate polymerase-based primer extension (see *CPMB UNIT 7.4A*; Htun and Johnston, 1992).

Interference and missing contact experiments both rely on chemical reagents to generate statistical populations or libraries of DNA that collectively contain a particular modification at various sites along a nucleotide sequence to block or remove potential contacts between DNA and proteins (Brunelle and Schleif, 1987; Wissmann and Hillen, 1991). For example, DMS methylation of guanine N7 (G N7) will not inhibit protein binding to the major groove except for the subpopulation of DNA that is modified within its recognition domain. The crucial sites required for binding are then identified from the product distribution of the protein-bound and free sequences (Fig. 6.6.2). Alternatively, this same methylation process can be used to induce depurination and delete certain interactions that stabilize protein-DNA assembly (Brunelle and Schleif, 1987). In this case, sequence recognition is identified by the subpopulation of apurinic DNA that exhibits reduced affinity for the protein of interest. Any abasic site has the additional potential to condense with neighboring lysine residues and form protein-DNA cross-links. This provides yet another tool for analyzing DNA-protein association (Mirzabekov et al., 1989; Bavykin and Pruss, 1997). For all of the methods described above, success depends on selection of the appropriate chemical reagents that modify DNA in a highly predictable, reproducible, and selective manner.

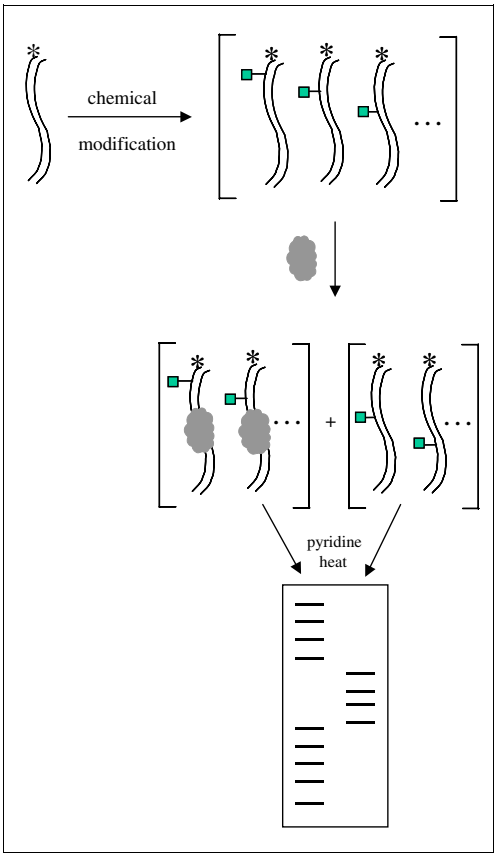

Figure 6.6.2 An interference assay for characterizing protein-DNA interactions.

INDIVIDUAL CHARACTERISTICS OF REAGENTS COMMONLY USED TO PROBE THE MAJOR GROOVE OF DNA

Despite the ever-growing number of reagents known to react in the major groove of DNA, none have maintained more prominence than DMS, DEPC, and $KMnO_4$. The popularity of these and most reagents is a function of their reliable specificity and ready availability. The following section provides a brief overview of these reagents, as well as OsO_4 and Br^-/HSO_5^-, and explores the origins and limitations of their selectivity. Specific examples illustrating concurrent application of these reagents are left for the final sections of this unit.

Dimethyl Sulfate (DMS)

The small and simple methylating agent DMS was among the first in a set of reagents frequently used to characterize DNA, and was central to many of the initial efforts in nucleotide sequencing, protection interference, and missing contact experiments. Application of DMS remains widespread due in part to this versatility. The predominant site of methylation in duplex DNA is the N7 position of guanine on the surface of the major groove (Singer and Grunberger, 1983). The resulting N7 methyl derivative is only metastable, and ultimately generates an abasic site that can be detected by its diagnostic fragmentation after treatment with piperidine and heat (Fig. 6.6.3). This chemistry is the basis for the now famous Maxam-Gilbert sequencing reaction for guanine (Maxam and Gilbert, 1980; also see *CPMB UNIT 7.5*). Methylation of G N7 does not inhibit polymerase chain extension, and thus piperidine-dependent strand scission is a necessary prerequisite for detecting this modification with the Klenow fragment of polymerase I (Saluz and Jost, 1989; Htun and Johnston, 1992). With the advent of thermostable polymerases like *Taq* polymerase, the heat of thermocycling is sufficient to fragment the methylated DNA and may now supersede the need for piperidine treatment (Brewer et al., 1990).

The same characteristics that made DMS appealing for sequencing have also made it attractive for many other applications. DMS modifies G N7 with equal proficiency in single- and double-stranded DNA and does not exhibit sequence-dependent activation or inhibition (Hartley, 1993). Consequently, a relatively homogeneous profile of methylation can be generated with DMS. This in turn facilitates the interpretation of all sequencing and footprinting experiments. The intrinsic reactivity of DMS is quite high, and, as with most reagents that act on DNA, precautions must be taken to avoid contact with it (see *CPMB UNIT 7.5*). The danger of DMS is further compounded by its neutral and lipophilic nature, two properties that are largely responsible for its desirable lack of sequence specificity.

The small size of DMS relative to DNA also provides it with unencumbered access to G N7, and the phosphoribose backbone neither shields the reagent from nor attracts it to particular regions or conformations of DNA. Other sites in DNA are also methylated by DMS, although at far lower efficiencies. For example, the N3 position of adenosine (A N3), located in the minor groove of duplex DNA, is subject to modification but reacts at a rate ~4-fold slower than that of G N7 (Singer and Grunberger, 1983). Other potential sites of alkylation are blocked by the formation of duplex DNA. The profile of methylation of single-stranded DNA is consistent with the nucleophilicity and electrostatic potential of the nitrogen heteroatoms (Pullman and Pullman, 1981). G N7 is significantly more reactive than the next most reactive position, A N1, and these are followed by A N7 > A N3 > G N3 (Singer and Grunberger, 1983). Theoretical and experimental investigations additionally confirm that A N7 is not nearly as nucleophilic as G N7 (Pullman and

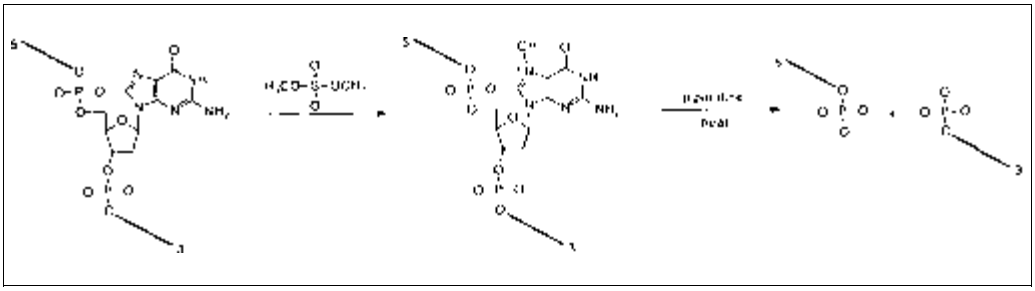

Figure 6.6.3 DMS-dependent methylation of G N7 and subsequent strand scission promoted by piperidine and heat.

Pullman, 1981; Singer and Grunberger, 1983). DMS was even used to map the nucleophilic sites within the pyrimidine nucleobases, despite their modest reactivity at the nucleoside level and even weaker reactivity when assembled into duplex DNA (Singer and Grunberger, 1983).

The preference of DMS for G N7 remains sufficient in most target sequences so that complications rarely arise from reaction at competing sites. Methylation is only effectively inhibited when the accessibility of G N7 is severely limited by drug, oligonucleotide, or protein binding in the major groove (Nielsen, 1990). This result in turn serves as the basis for interference studies that help to localize binding sites in DNA. Quadruplexes and other unusual structures of DNA that involve coordination of G N7 also exhibit protection from DMS (Sen and Gilbert, 1988; Williamson et al., 1989; Fig. 6.6.4).

Two caveats that may effect data evaluation were noted in a review by Nielsen (1990), and are well worth repeating. First, DMS has the potential to disrupt DNA-ligand interaction by methylation of the ligand in competition with its methylation of DNA. Second, proteins have the potential to increase the local concentration of DMS since they may establish lipophilic binding pockets in the vicinty of DNA. The lack of charge on DMS also renders this reagent relatively insensitive to the electrostatic properties of DNA and the ionic strength of solution (Wurdeman and Gold, 1988). This is in direct contrast to the nature of other alkylating agents, such as nitrogen mustards, that modify G N7 through a cationic intermediate and consequently demonstrate electrostatic affinity for DNA (Hartley et al., 1990).

Diethylpyrocarbonate (DEPC)

A N7 is the target most commonly associated with reaction of DEPC (Peattie, 1979; Nielsen, 1990). Initial carbethoxylation of N7 leads to cleavage of the imidazole ring and subsequent alkaline lability of the phosphoribose backbone (Fig. 6.6.5). Reaction profiles of DEPC are therefore typically examined after piperidine treatment of the modified DNA. Interest in DEPC as a probe for DNA originated from its earlier applications in determining RNA sequence and structure (Peattie, 1979; Peattie and Gilbert, 1980), which in turn evolved from its even earlier use as a histidine-specific reagent for protein modification (Lundblad, 1995). This ability to modify proteins should now remain a concern when using DEPC to map protein-DNA interactions. The conformational specificity of DEPC as characterized with RNA is very sensitive to base stacking and is severely inhibited in helical structures (Peattie and Gilbert, 1980; Ehrsemann et al., 1987; Weeks and Crothers, 1993). Single-stranded and nonhelical regions provide the most accessible targets of DEPC within RNA. Equivalent specificity was detected for DNA, although some reaction was additionally noted within duplex DNA (Herr et al., 1982; Furlong and Lilley, 1986).

Adenine residues are not the exclusive target of DEPC. Guanine residues, particularly those with high solvent exposure at N7, may also react (Herr, 1985; Johnston and Rich, 1985; Runkel and Nordheim, 1986; Scholten and Nordheim, 1986). Early studies indicated that both pyrimidine and purine nucleosides were subject to carbethoxylation by DEPC (Leonard et al., 1971; Vincze et al., 1973), but only purines appear to maintain an observable reactivity in polynucleotides (Nielsen, 1990). Within helical DNA, DEPC generally favors modification of A over G when the accessibility of their N7 positions is similar (Herr, 1985). The origin of this selectivity has not yet been determined, and is certainly not based on nucleophilicity. Otherwise, guanine would have been most reactive as illustrated with DMS. Neither the general electrostatic nature of DNA

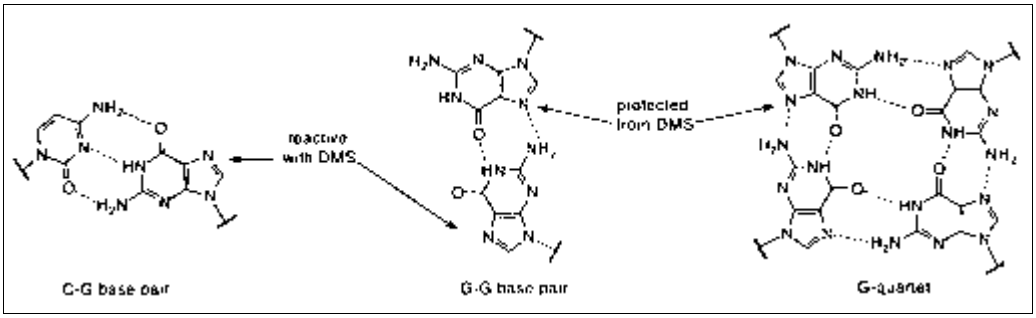

Figure 6.6.4 DMS reaction and protection for G-C and G-G base pairs and G-quartets.

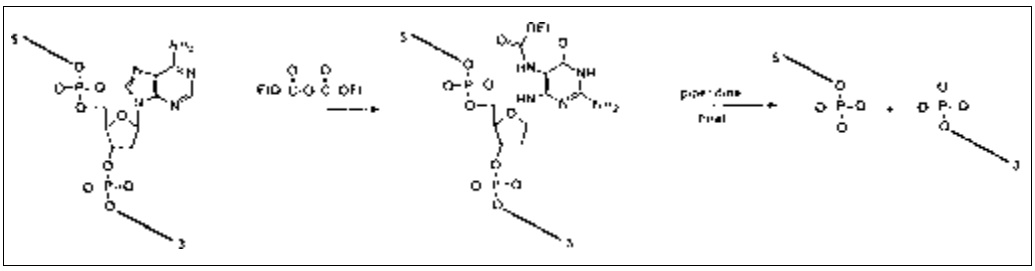

Figure 6.6.5 DEPC-dependent modification of A N7 and subsequent strand scission promoted by piperidine and heat.

nor the ionic conditions of reaction greatly affect the specificity of DEPC as expected for a neutral reactant (Klysik et al., 1990; Nejedlý et al., 1998). The most predictable determinant for modification of A appears simply to be the steric accessibility of its N7 position.

Potassium Permanganate ($KMnO_4$) and Osmium Tetroxide (OsO_4)

Both reagents selectively oxidize the C5, C6 double bond of thymine residues (Fig. 6.6.6) and support convenient methods for DNA sequencing and conformational analysis (Nielsen, 1990). The lack of charge on OsO_4 minimizes its sensitivity to the electrostatic properties of DNA and may simplify interpretation of modification data relative to those based on $KMnO_4$. However, OsO_4 is somewhat volatile and quite hazardous (Paleček, 1992a), and is not applied as frequently as the safer alternative, $KMnO_4$. Even though the ultimate oxidant MnO_4^- is charged, it remains cell-permeable for in vivo studies (Sasse-Dwight and Gralla, 1989). The anionic characteristic also suppresses reaction with DNA due to electrostatic repulsion of DNA, despite the enduring focus in the general literature on the importance of sterics (Hänsler and Rokita, 1993).

Oxidation of duplex DNA with $KMnO_4$ and OsO_4 is greatly inhibited relative to that of single-stranded DNA, and consequently these reagents are very useful for identifying regions of unusual and nonhelical structure. Perturbations of duplex DNA caused by association of proteins and drugs are also often sufficient for stimulating a local hyperreactivity (Fig. 6.6.2). Even minor distortions in DNA conformation resulting from single base mismatches can be detected by $KMnO_4$ and OsO_4 (Cotton et al., 1988; Roberts et al., 1997; Lambrinakos et al., 1999). The *cis* diol product of T oxidation is conveniently detected by either strand scission induced by piperidine and heat (Friedman and Brown, 1978; Rubin and Schmid, 1980) or termination of primer extension catalyzed by a variety of common DNA polymerases (Ide et al., 1985; Borowiec et al., 1987; Clark and Beardsley, 1987).

The specificity and potential utility of MnO_4^- was noted very early in the quest for

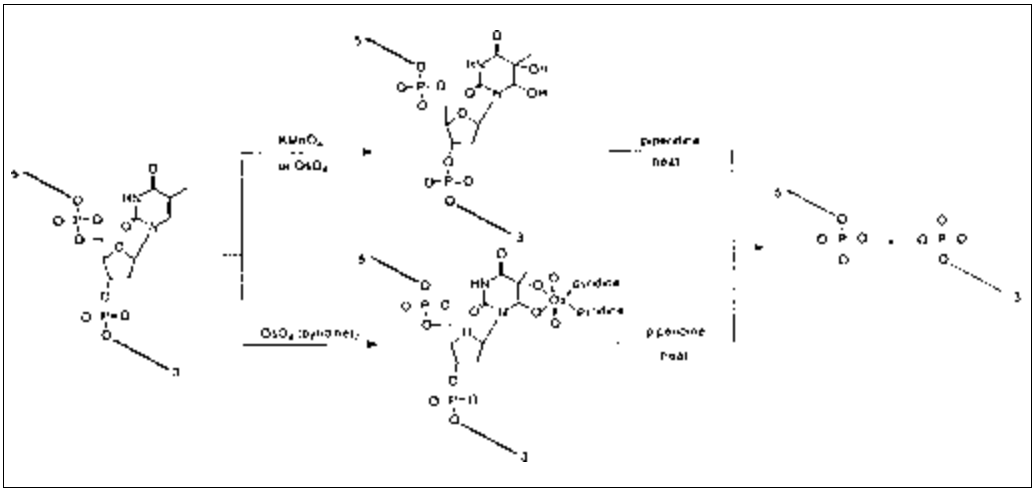

Figure 6.6.6 $KMnO_4$- and OsO_4-dependent oxidation of thymine and subsequent strand scission promoted by piperidine and heat.

chemical probes of nucleic acids. Access to the target C5, C6 of T is an understandable requirement for efficient reaction, and hence may explain the preference for single- versus double-stranded DNA (Hayatsu and Ukita, 1967). Residues within duplex DNA that became sensitive to MnO_4^- after supercoiling or binding to a protein were originally considered indicative of helix distortions and base unpairing (Borowiec et al., 1987; O'Halloran et al., 1989). However, the enhanced exposure of T anticipated from chemical modification is not always evident in the crystal structure of protein-DNA complexes (Bochkarev et al., 1998). In some cases, only subtle changes in conformation have appeared responsible for the high reactivity of thymine residues. These unusual sites of modification might reflect protein-dependent shielding of the anionic charge of DNA from the anionic reagent MnO_4^- (Hänsler and Rokita, 1993). Permanganate oxidation of duplex DNA can be accelerated 25-fold by merely increasing the ionic strength of the reaction solution from 0.1 to 4.0 M (Hänsler and Rokita, 1993). Under these conditions, the high concentration of cations effectively diminishes charge repulsion between the reactants. This dependence on ionic strength is not an inherent characteristic of MnO_4^- since the oxidation of neutral thymidine is enhanced only 1.3-fold in a comparable study (Hänsler and Rokita, 1993). Similarly, oxidation of DNA by OsO_4 is unaffected by ionic strength (Nejedlý et al., 1998). Permanganate then serves as an example of how reagents with a charge may respond to both steric and electrostatic properties of DNA.

The specificity of MnO_4^- for T was first demonstrated using mononucleotides (Hayatsu and Ukita, 1967). Under conditions that consumed 95% of TMP, the purines dGMP and dAMP were nearly inert, and the pyrimidine dCMP was only marginally reactive. Even uridine 5′-phosphate that only lacks the 5-methyl of TMP oxidized at a rate ~10-fold slower than TMP. The resulting product of oxidation, the *cis* 5,6-diol of T, readily hydrolyzes to an abasic site (Howgate et al., 1968). These favorable characteristics led MnO_4^- to become a standard reagent for chemical sequencing of DNA (Rubin and Schmid, 1980; McCarthy, 1989; Williamson and Celander, 1990). Occasionally, background oxidation of duplex DNA has been observed particularly at guanine residues (McCarthy et al., 1990; McCarthy and Rich, 1991). The origin of this has not yet been identified, and controversy remains on whether or not 8-oxoguanine is a product of this minor pathway (Akman et al., 1990; Nawamura et al., 1994). Perhaps the background reactions are a result of prior modification or contamination of the parent DNA. For example, 8-oxoguanine residues and their neighboring bases, as well as guanine side products encountered during oligonucleotide synthesis, are all targets of MnO_4^- oxidation (Yeung et al., 1988; Koizume et al., 1998).

The reactivity of OsO_4 is uniquely activated by the presence of tertiary amine ligands, and thus this reagent is often used in the presence of pyridine or 2,2′-bipyridine (Paleček, 1992a). Such ligands dramatically stabilize the osmium ester intermediate (Fig. 6.6.6). Unlike MnO_4^- and OsO_4, which generate the diol derivative of thymine, the OsO_4-pyridine complex proceeds only to the cyclic ester (Neidle and Stuart, 1976). This species hydrolyzes in a manner equivalent to the diol under standard alkaline conditions to yield strand fragmentation. Alternatively, a primer-extension assay can be used to identify the sites of modification. The conformational specificity of oxidation can be controlled in part by the choice of amine used to coordinate to the osmium (Paleček, 1992a). The ultimate extension of this strategy is illustrated by the sequence-directed oxidation of a single thymine using an osmium complex derived from a bipyridine-oligonucleotide conjugate (Ford et al., 1981; Nakatani et al., 2000).

Bromide (Br^-) with Monoperoxysulfate (HSO_5^-)

A very promising method of detecting cytosine residues in nonhelical DNA has been recently developed and relies on generation of bromine (Br_2) in situ by oxidation of Br^- in the presence of HSO_5^- (Ross and Burrows, 1996; Fig. 6.6.7). Extrahelical C residues exhibit a 10-fold increase in reactivity above a low background level in duplex DNA. In single-stranded structures, modification of C is minimally 4-fold more efficient than that of T, G, or A. Preferential reaction of C is also observed after equivalent addition of Br_2, although its selectivity is reduced compared to that of Br^-/HSO_5^- (Ross and Burrows, 1996). Both conditions result in the intermediate formation of 5-bromo C. This is susceptible to further addition by Br_2, which in turn induces DNA strand fragmentation after treatment with piperidine and heat (Ross and Burrows, 1996, 1997). As more investigators begin to explore the utility of Br^-/HSO_5^- (Stevens and Glick, 1999; Kasparkova et al., 2000; Kostrhunova

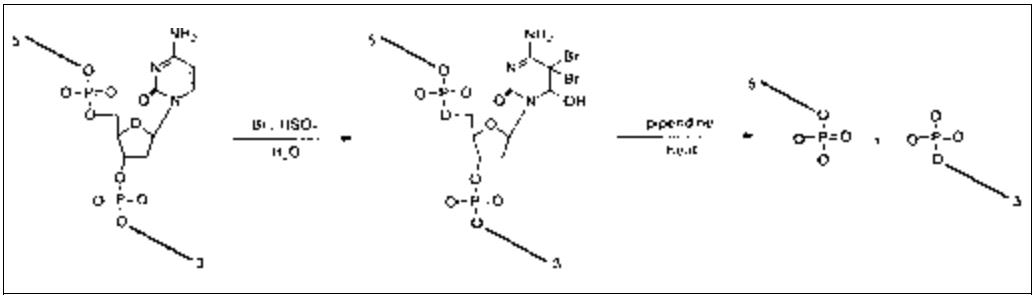

Figure 6.6.7 Oxidation of C by Br^-/HSO_5^- and subsequent strand scission promoted by piperidine and heat.

and Brabec, 2000), this technique will likely become the MnO_4^- or OsO_4 equivalent for C. Two alternative reagents, hydroxylamine (Rubin and Schmid, 1980; Johnston and Rich, 1985; Johnston, 1992) and bisulfite (Hayatsu, 1976; Gough et al., 1986), have been known for many years to react with C in a conformation-selective manner, but their use has been very limited. This lack of popularity may be due in part to the very high concentration of reagents (2 M) that is required for sufficient modification (Johnston and Rich, 1985; Gough et al., 1986). Interest in bisulfite has recently revived since its selectivity for C over 5-methyl C has provided a convenient method for mapping methylation patterns of CpG islands in DNA (Rother et al., 1995; Kinoshita et al., 2000). Bisulfite reaction with C has also become central to a protocol for mapping chromatin in vivo in concert with methyltransferases (Kladde and Simpson, 1996).

APPLYING CHEMICAL REAGENTS TO PROBE THE MAJOR GROOVE OF DNA

Sample investigations based on chemical approaches to characterizing DNA structure, and particularly its major groove, are described below. The goal of this section is to illustrate the range of information that is made available by these methods, rather than provide a comprehensive survey of all major advances that have occurred since previous review of this subject (Nielsen, 1990; Tullius, 1991; Lilley, 1992; Chow and Barton, 1992). Topics are organized into four sections, DNA conformation, drug-DNA association, protein-DNA association, and in vivo footprinting. Most examples involve the use of multiple reagents, since definitive conclusions are often difficult to establish with only a single reagent. Complementary techniques of footprinting (protection) and interference or missing contacts are also often performed concurrently to substantiate the results of individual analyses. Although chemical modification does not offer the highest atomic resolution, it represents a very expedient method for defining key structural features of DNA and identifying the functional groups responsible for recognition and binding to proteins and drugs.

Conformational Analysis of Duplex DNA

Uniform double-helical DNA typically yields an equally uniform pattern of modification, and even minor perturbations of groove dimensions or base pairing have the potential to promote or inhibit reaction of conformation-specific probes. For example, biological methylation of A to form N^6-methyl A appears to weaken A-T pairing and stacking in duplex DNA, as detected by the greater susceptibility of its N7 position to reaction with DEPC (Guo et al., 1995). Numerous investigators have also used DEPC to characterize the properties of A-tract DNA. The N7 position of A is more reactive in A-tracts than in canonical duplex DNA. This is consistent with the wider major groove and narrower minor groove associated with A-tract DNA (McCarthy et al., 1990, 1993; Nejedlý et al., 1998). In contrast, the complementary T-tract does not exhibit high reactivity with either OsO_4-bipyridine or MnO_4^- and suggests that the widened major groove still maintains considerable steric and electrostatic repulsion (McCarthy et al., 1993; Nejedlý et al., 1998). Some deviations from this general reaction profile, and hence helical structure of DNA, have additionally been noted at certain A-tract junctions and in natural variants of A-tract DNA (McCarthy et al., 1993; Chang et al., 1994; Nejedlý et al., 1998).

The sensitivity of DEPC, OsO_4-pyridine, and MnO_4^- to nucleotide stacking extends to parallel-stranded duplex DNA (Klysik et al., 1990), and its helix-coil transition may be monitored by exposure to OsO_4-pyridine. An-

tiparallel DNA forming a left-handed Z helix represents another highly unusual duplex structure that has been examined by chemical probes. DEPC readily reacts with the N7 positions of both A and G residues within Z-helical domains (Herr, 1985; Johnston and Rich, 1985; Runkel and Nordheim, 1986). This modification is relatively independent of base stacking, since the N7 position in Z DNA is oriented towards the outer edge of the helix and maintains much greater accessibility than it does in right-handed B DNA (Pullman and Pullman, 1981). B-Z and Z-Z helical junctions are readily modified by a variety of reagents including OsO_4 and MnO_4^- in a manner consistent with a region of high disorder or conformational dynamics (Nejedlý et al., 1985; Falazka et al., 1986; Jiang et al., 1991).

All of the reagents introduced above except for DMS are quite useful in identifying extrahelical or weakly stacked bases within standard duplex DNA. DMS is insensitive to these perturbations because both single- and double-stranded DNA are methylated at G N7 with equal efficiency (Hartley, 1993). However, subtle changes in helical conformation caused by noncanonical structures such as base mismatches containing T may be detected by oxidation with MnO_4^- in the presence of tetralkylammonium salts (Gogos et al., 1990; Roberts et al., 1997; Lambrinakos et al., 1999). Addition of this type of salt appears to enhance selectivity for mismatches (Cotton, 1989). These conditions now serve as the basis for a protocol entitled "chemical cleavage of mismatch" for detecting point mutations (Gogos et al., 1990; Roberts et al., 1997; Lambrinakos et al., 1999). Interestingly, reaction is not limited to mispaired T. MnO_4^- additionally seems to oxidize some mismatched G and C residues, and both MnO_4^- and OsO_4 oxidize normally paired T residues adjacent to mismatched bases (Cotton and Campbell, 1989; Lambrinakos et al., 1999).

Single-stranded regions of DNA formed by cruciform or hairpin structures are readily identified by conformational probes as well, and the presence of T, C, and A in these structures is typically identified by reaction with MnO_4^-, Br^-/HSO_5^-, and DEPC, respectively (Scholten and Nordheim, 1986; Hänsler and Rokita, 1993; Stevens and Glick, 1999). These reagents have also been successfully applied to characterization of four-way helical junctions (Webb and Thomas, 1999), strand displacement and invasion by peptide nucleic acids (Egholm et al., 1995; Armitage et al., 1997), and equilibria of single-, double-, and triple-stranded species formed by naturally occurring triplet base repeats (Mäueler et al., 1998; Fig. 6.6.8). Once again, methylation of G N7 by DMS cannot discriminate between these forms of DNA. An alternative reagent for G N7, not discussed in this review but sensitive to secondary structure, involves nickel- or cobalt-dependent oxidation of G in the presence of HSO_5^- (*UNIT 6.4*).

The first indication that G-rich telomeric sequences could form four-stranded structures was based on their unusual pattern of modification with DMS, MnO_4^-, and DEPC (Sen and Gilbert, 1988; Williamson et al., 1989; Venczel and Sen, 1993; Balagurumoorthy and Brahmachari, 1994). In this case, DMS was particularly useful, since the quadruplex assembled into a so-called G-quartet that is stabilized by Hoogsteen base pairs that involve G N7 (Sen and Gilbert, 1988; Williamson et al., 1989; Fig. 6.6.4). Thus, formation of the G-quartet inhibited methylation at this site and, conversely, methylation inhibited G-quartet formation. Nucleotides linking the G-rich regions were targets of DEPC and MnO_4^- reaction when looped between G-quartets, but were inert when stacked in the standard B duplex.

Drug-DNA Association

The footprint of a low-molecular-weight drug on DNA often extends over only a few

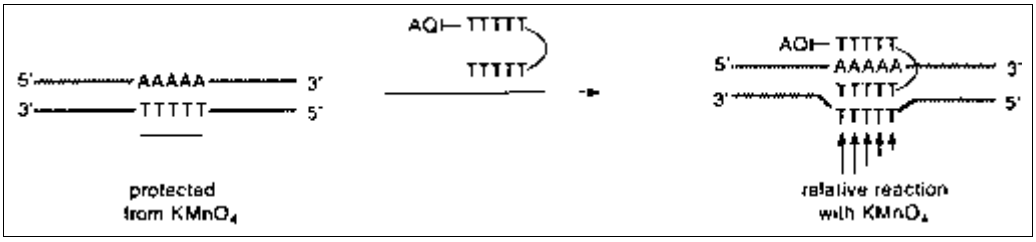

Figure 6.6.8 Strand displacement increases exposure of target sequences to chemical probes. A peptide nucleic acid analog based on thymine and an anthraquinone intercalator (AQI) was used to recognize an A_5 sequence (Armitage et al., 1997).

base pairs, and consequently chemical modification data are often difficult to interpret during the initial stages of characterizing drug-DNA interactions. Instead, reagents are most useful when applied to well-defined oligo- or polynucleotide model systems that are designed to address specific questions on drug binding. The reagents highlighted in this unit are also not typically the first employed, since major-groove recognition is rather rare for either synthetic or natural compounds. Two notable exceptions to this generalization are triplex-forming oligonucleotides that are currently under development as gene-targeted drugs (Miller, 1996; Giovannangeli and Hélène, 1997; Fox, 2000) and cisplatin derivatives that have already proven highly successful in treatment of certain cancers (Jamieson and Lippard, 1999; Wong and Giandomenico, 1999). Assembly of a third DNA strand into the major groove of duplex DNA protects A N7 and G N7 from DEPC and DMS reaction, respectively (Collier et al., 1991; Beal and Dervan, 1992). Similarly, platinum coordinates to DNA through G N7, and hence protects this site from DMS-dependent methylation (Sip et al., 1992; Kasparkova et al., 2000). Interstrand cross-linking at d(GC/CG) sites by platinum does not appear to cause significant unstacking of DNA, at least as indicated by the reaction profile of DEPC and OsO_4 (Sip et al., 1992). Structural perturbations are limited to the two C residues directly adjacent to the platinum, as identified by reaction of hydroxylamine. Intrastrand cross-linking by cisplatin and a related distamycin conjugate induced only a localized hyperreactivity of MnO_4^-, DEPC, and Br^-/HSO_5^-, and this was consistent with an asymmetric and local unwinding of DNA (Marrot and Leng, 1989; Kasparkova et al., 2000; Kostrhunova and Brabec, 2000; Fig. 6.6.9).

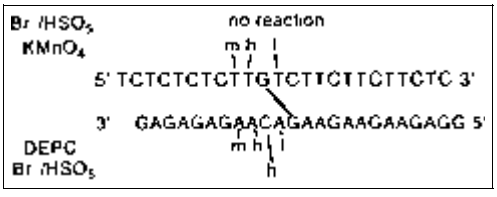

Figure 6.6.9 Local distortion of duplex DNA induced by a dinuclear platinum complex. Relative reaction is designated as high (h), medium (m), and low (l). Adapted from Kasparkova et al. (2000) with permission from The American Society for Biochemistry and Molecular Biology.

A variety of intercalating agents such as ethidium, 9-aminoacridine, and *N,N*-di(9-acridinyl)spermidine stimulate unwinding of helical DNA as determined in part by reaction with DEPC and MnO_4^- (Jeppesen and Nielsen, 1988). These same probes similarly helped to identify the sequence-dependent binding properties of the natural bisintercalator echinomycin (Bailly et al., 1994). Even drugs such as bleomycin that do not directly interact with the major groove may still cause sufficient structural distortion of DNA to affect its subsequent reactivity (Fox and Grigg, 1988). In particular, footprinting by DEPC and MnO_4^- suggests that bleomycin enhances exposure of the nucleotide directly 3′ to its binding site. As expected, DMS is generally insensitive to such binding, since no steric barrier is created around G N7.

Protein-DNA Association

The great majority of protection and interference experiments designed to probe protein-DNA interactions within the major groove rely on DMS and MnO_4^-. All reagents have the potential to react with protein in a competitive manner, but the greatest number of complications likely arise from alternative reagents such as DEPC and OsO_4 (Paleček, 1992b; Lundblad, 1995). Consequently, DEPC and OsO_4 are applied with less frequency to study protein-DNA complexes (Dobi and Agoston, 1998; Mäueler et al., 1998). Investigations based on Br^-/HSO_5^- have only just begun, and therefore their potential to map protein-DNA interactions is not yet known. However, these conditions have the potential to oxidize a variety of amino acid side chains that might disrupt protein-DNA association.

DMS and MnO_4^- alone can still provide considerable information on DNA-protein interactions, since their reaction specificities are quite complementary. Methylation of G N7 by DMS is unaffected by the helical conformation of DNA, but can be suppressed by loss of major groove accessibility due to protein binding. In contrast, oxidation of T by MnO_4^- is suppressed in helical DNA and activated by relaxation of its conformational and electrostatic constraints due to protein binding. Such effects were observed after *lac* repressor bound to its operator sequences (Borowiec et al., 1987). DMS protection was evident at two repressor binding sites, while MnO_4^- reaction was enhanced in an intervening AT-rich sequence. Similarly, an open transcription complex formed by RNA polymerase and MerR protein bound with Hg^{2+} conferred hypersensitivity to MnO_4^- at nine T

residues along a 14-nucleotide sequence (O'Halloran et al., 1989). In contrast, the Hg^{2+}-MerR protein complex in the absence of the polymerase appeared to unwind duplex DNA without affecting MnO_4^- reaction, and the polymerase in the absence of the MerR complex induced MnO_4^- reaction at only five T residues (O'Halloran et al., 1989; Ansari et al., 1992). Each of the intermediate DNA-protein complexes were characterized by their unique patterns of DMS protection. Surprisingly, a small number of G residues also became hyperreactive to DMS. Since DMS is insensitive to DNA unwinding, reaction efficiency might have increased due to the accumulation of DMS in hydrophobic pockets of the complexes.

Open transcription complexes formed at other DNA sequences also exhibit hyperreactivity with MnO_4^- (Jeppesen and Nielsen, 1989), and this characteristic has since served as the basis for determining which amino acids within a series of σ70 mutants help to stabilize the open complex (Fenton et al., 2000). In addition, quantitative analysis of the DNA fragments generated in related experiments yielded information on the population distribution of alternative protein-DNA conformers (Tsodikov et al., 1998). The hyperreactivity observed for these systems may originate in part from a protein-dependent flipping of adenosine bases out of the DNA helix to expose their complementary T residues (Fenton et al., 2000). Related reaction is evident when T is held in an extrahelical position by enzymes that utilize a base-flipping mechanism during their processing of DNA (Serva et al., 1998; Reddy and Rao, 2000; Fig. 6.6.10). DMS-dependent methylation generates an equally diagnostic pattern of hyper- and hyporeactive G residues within a complex of MutY and DNA, which also involves nucleotide flipping (Chepanoske et al., 1999).

At least for MnO_4^-, the basis of protein-dependent hyperreactivity is not always so evident. Two protein-DNA complexes characterized by X-ray crystallography do not reveal the extensive unpairing of T at sites that were previously shown to have a high sensitivity to MnO_4^- (König et al., 1996; Bochkarev et al., 1998). A single T proximal to the pseudo-dyad axis of nucleosomes was similarly found to be hyperreactive, but not necessarily extrahelical (Fitzgerald and Anderson, 1999). Perhaps the lysine-rich histone tails were instead able to attenuate the electrostatic repulsion of the anionic reagent and DNA and allow for efficient oxidation of T (Hänsler and Rokita, 1993).

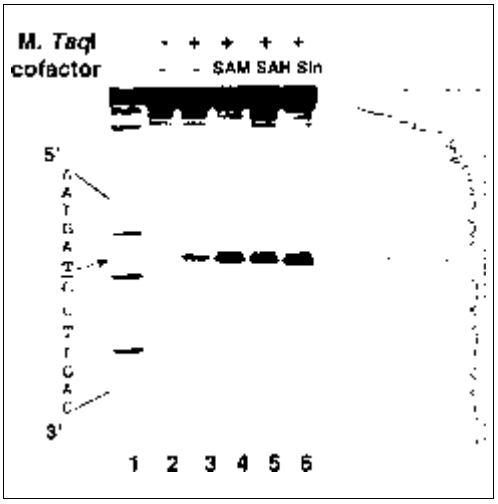

Figure 6.6.10 Base flipping can be detected by chemical modification. $KMnO_4$ was used to detect an extrahelical T formed by duplex DNA in the presence and absence of DNA adenine-N^6 methyltransferase (M. *Taq*I), *S*-adenosyl methionine (SAM), *S*-adenosyl homocysteine (SAH), and sinefungin (Sin; adenosyl ornithine). Densitometry traces of lanes 2, 3, and 4 are shown to the right. Reprinted from Serva et al. (1998) with permission from Oxford University Press.

Just as a protein may influence chemical modification of DNA, so too may chemical modification of DNA affect its association with a protein (Fig. 6.6.2). Accordingly, an enhancer factor R from Epstein-Barr virus could have been characterized through its ability to protect a region of DNA from DMS-dependent methylation, but, instead, methylation of G N7 was used to determine which sites block binding of the enhancer (Gruffat et al., 1990). Oxidation of T to its glycol derivative (Fig. 6.6.6) has similarly been used for interference analysis of GCN4 binding to the major groove of DNA (Pu and Struhl, 1992). This type of interference experiment represents a convenient and complementary alternative to the protection studies described above.

Together, these approaches can establish a compelling model for specific protein-DNA complexes and even help discover new DNA-protein systems. For example, an 80-bp region of DNA and two associated nuclear factors that are involved in transcription control of histone $H1^0$ were identified through a combination of techniques including chemical footprinting, UV cross-linking, DMS interference, and site-directed mutagenesis (Breuer et al., 1993). When studies are based solely on interference

assays, a series of reagents are often employed. In this manner, results based on DMS and DEPC were sufficient to detect distinct protein-DNA contacts with two consensus-sequence variants recognized by the M-lysozyme downstream enhancer (Nickel et al., 1995). The importance of using multiple reagents is further demonstrated by investigations on a series of hormone-responsive elements. DMS interference suggested that both the glucocorticoid and estrogen receptors form similar contacts to G N7, but MnO_4^- interference was able to determine that only the glucocorticoid receptor interacts with T (Truss et al., 1991; Fig. 6.6.11).

Missing contact probing offers yet another complementary approach for examining protein-DNA complexes. In this case, chemical modification removes, rather than masks, functional groups of DNA that have the potential to interact with protein. A population of DNA lacking individual guanine bases can be generated by treatment with DMS and can be used to screen for individual G residues that are critical for stabilizing protein-DNA interactions. This was first applied to investigations on the λ phage repressor protein (Brunelle and Schleif, 1987) and has more recently been combined with DMS and $KMnO_4$ interference studies to ascertain the DNA binding properties of a nitrogen regulatory protein (Feng et al., 1993). Missing contact experiments are not limited to depurination of G by DMS. Depurination of G + A by formic acid, depyrimidation of T + C by hydrazine, and depyrimidation of T by $KMnO_4$ have all been used to identify protein-DNA interactions (Brunelle and Schleif, 1987; McBoom and Sadowski, 1994). Any one of these procedures may be adequate to address a particular question on structure, but when used in concert with protection and interference analysis, a broad range of structural characteristics may be described in the absence of crystallography or NMR.

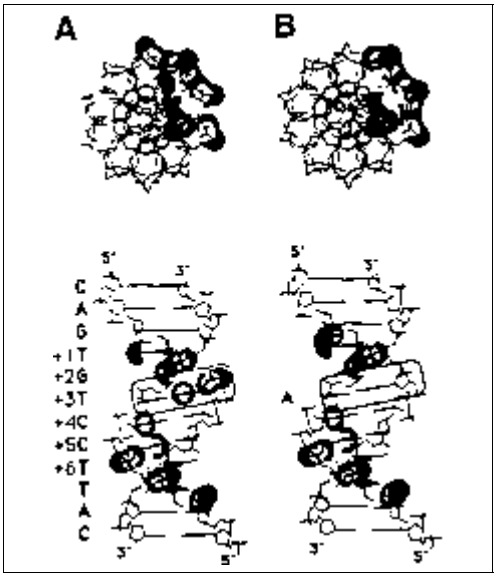

Figure 6.6.11 Protein contact sites can be mapped onto helical models of duplex DNA. Interference assays were used to differentiate DNA recognition by (**A**) the glucocorticoid receptor with a half-site of its responsive element and (**B**) the estrogen receptor with a half-site of its equivalent responsive element. DMS, $KMnO_4$, and ethylnitrosourea were used to probe N7 of G, C5, C6 of T, and phosphate oxygens of the backbone, respectively. Reprinted from Truss et al. (1991) with permission from the American Society for Microbiology.

Applications of Chemical Modification In Vivo

The vast majority of studies based on chemical modification are performed in vitro for obvious reasons including convenience and simplicity. However, questions regarding biological relevance can linger in the absence of complementary data for equivalent systems in vivo. One of the many procedures available to help forge the necessary connection to cellular conditions is none other than chemical modification. Such an approach might be initially dismissed out of concerns about cell permeability and target specificity, but numerous laboratories have successfully footprinted DNA in vivo.

One of the first examples utilized methylation of G N7 by DMS to detect regulatory proteins binding to the *lac* operon (Nick and Gilbert, 1985). DNA modification depended on the ability of this neutral and low-molecular-weight organic reagent to diffuse into bacterial cells. Subsequent mapping of the DNA reaction originally entailed chromatin isolation, restriction, separation, and Southern blotting. Primer extension soon replaced blotting (Borowiec and Gralla, 1986), and more recently ligation-mediated PCR and use of AlkA protein, a glycosylase that hydrolyzes DNA at N^3-methyl A and N^7-methyl G, have been adopted to enhance the detection of chemical probing in vivo (Szabó et al., 2000).

Despite the charge and high reactivity of MnO_4^-, this oxidant has similarly been used to examine complexes of *lac* promoter and RNA polymerase within *Escherichia coli* (Sasse-Dwight and Gralla, 1989). The specificity of MnO_4^- for single-stranded DNA is especially

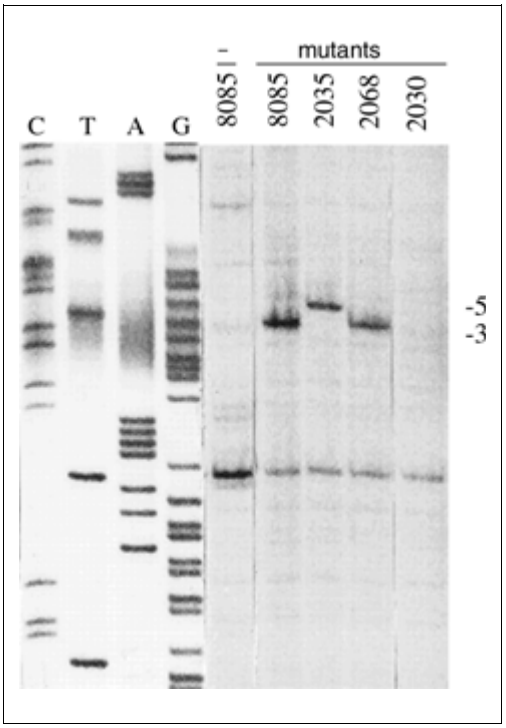

Figure 6.6.12 In vivo footprinting of open complexes formed by RNA polymerase and various mutant promoter complexes using $KMnO_4$. Reprinted from Barrios et al. (1998); copyright (1998) National Academy of Sciences, U.S.A.

suited for detecting the open complex formed with RNA polymerase. Complexes containing alternative promoters and sigma factors have also been investigated in vivo with both DMS and MnO_4^- in relationship to transcription control for growth under anaerobic and aerobic conditions (Morett and Buck, 1989; Barrios et al., 1998; Fig. 6.6.12).

OsO_4 and its bipyridine complex provide alternative reagents for probing T in vivo (Paleček, 1992b). Although OsO_4 is often used as a general stain for cells, DNA modification can be accomplished with concentrations lower than those needed for staining. The most notable success of OsO_4 in vivo has been the detection of hyperreactive T residues that may result from the formation B-Z or Z-Z helical junctions within *E. coli* (Rahmouni and Wells, 1989). While these examples have certainly demonstrated the potential of chemical modification in vivo, widespread enthusiasm for this approach will likely depend on future development of reagents with much greater selectivity.

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