precursors, with different sized introns and ca. 10% amino acid variation in the protein-coding regions.

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Registry No. DNA (*Catostomus commersoni* vasotocin VT-1 messenger RNA complementary), 125048-68-2; DNA (*Catostomus commersoni* vasotocin VT-2 messenger RNA complementary), 125048-69-3; vasotocin VT-1 (*Catostomus commersoni* precursor reduced), 125048-71-7; vasotocin VT-2 (*Catostomus commersoni* precursor reduced), 125048-72-8; isotocin (*Catostomus commersoni* reduced), 124920-92-9; vasotocin (*Catostomus commersoni* reduced), 17451-88-6; isotocin, 550-21-0; vasotocin, 9034-50-8.

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Stabilization of the Topoisomerase II–DNA Cleavage Complex by Antineoplastic Drugs: Inhibition of Enzyme-Mediated DNA Religation by 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide†

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ABSTRACT: In order to elucidate the mechanism by which the intercalative antineoplastic drug 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) stabilizes the covalent topoisomerase II–DNA cleavage complex, the effect of the drug on the DNA cleavage/religation reaction of the type II enzyme from *Drosophila melanogaster* was examined. At a concentration of 60 μM, *m*-AMSA enhanced topoisomerase II mediated double-stranded DNA breakage ~5-fold. Drug-induced stabilization of the enzyme–DNA cleavage complex was readily reversed by the addition of EDTA or salt. When a DNA religation assay was utilized, *m*-AMSA was found to inhibit the topoisomerase II mediated rejoining of cleaved DNA ~3.5-fold. This result is similar to that previously reported for the effects of etoposide on the activity of the *Drosophila* enzyme [Osheroff, N. (1989) *Biochemistry 28*, 6157–6160]. Thus, it appears that structurally disparate classes of topoisomerase II targeted antineoplastic drugs stabilize the enzyme's DNA cleavage complex primarily by interfering with the ability of topoisomerase II to religate DNA.

Topoisomerase II is a ubiquitous enzyme that is essential for the viability of eukaryotic cells (Vosberg, 1985; Wang, 1985; Osheroff, 1989a). Fundamental to the enzyme's physiological DNA strand passage reaction is its ability to create and religate double-stranded breaks in the nucleic acid backbone (Vosberg, 1985; Wang, 1985; Osheroff, 1989a).

In addition to its important cellular functions, topoisomerase

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II is also the primary cellular target for a wide variety of clinically relevant antineoplastic agents (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). The chemotherapeutic actions of these drugs correlate with their abilities to stabilize the covalent topoisomerase II–DNA cleavage complex (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). Thus, as monitored in vitro, topoisomerase II targeted drugs shift the equilibrium of the enzyme's DNA cleavage/religation cycle toward the cleavage event.

Topoisomerase II targeted antineoplastic drugs can be grouped into two general categories on the basis of their DNA binding properties: those which bind to but are nonintercalative with respect to DNA and those which are strongly intercalative (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). Representatives of the first group include the epipodophyllotoxins (etoposide and tenoposide) (Ross et al., 1984; Chow et al., 1988), while those of the second include drugs such as 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA),[1] ellipticine, and adriamycin (Waring, 1981; Wilson et al., 1981). Recently, the drug etoposide has been shown to stabilize the DNA cleavage complex of *Drosophila melanogaster* topoisomerase II (at least in part) by inhibiting the enzyme's ability to religate its cleaved DNA reaction intermediate (Osheroff, 1989b). Comparable studies have yet to be reported for other antineoplastic agents. Considering the major structural differences which exist between the many classes of topoisomerase II targeted drugs, it is not known whether the actions of etoposide can be extrapolated to the intercalative drugs.

Therefore, in order to determine whether different classes of antineoplastic drugs act in a similar fashion, the effect of *m*-AMSA on the DNA cleavage/religation equilibrium of the *Drosophila* enzyme was characterized. As previously shown for etoposide (Osheroff, 1989b), *m*-AMSA was found to inhibit the DNA religation reaction of topoisomerase II. Thus, it appears that structurally disparate classes of topoisomerase II targeted drugs stabilize the enzyme's DNA cleavage complex by a common kinetic mechanism.

Experimental Procedures

DNA topoisomerase II was purified from the nuclei of *Drosophila melanogaster* Kc tissue culture cells by the procedure of Shelton et al. (1983). Negatively supercoiled bacterial plasmid pBR322 (Bolivar et al., 1977) was obtained from *Escherichia coli* by a Triton X-100 lysis followed by double banding in cesium chloride–ethidium bromide gradients (Maniatis et al., 1982). *m*-AMSA (NSC-249992) was the generous gift of Dr. Yves Pommier, Laboratory of Molecular Pharmacology, NCI. The drug was stored at −20 °C as a 10 mM solution in DMSO. Tris and ethidium bromide were obtained from Sigma; analytical reagent grade $CaCl_2 \cdot H_2O$ and $MgCl_2 \cdot 6H_2O$ were from Fisher, and SDS and proteinase K were from E. Merck Biochemicals. All other chemicals were analytical reagent grade.

Cleavage of DNA by Topoisomerase II. All DNA cleavage reactions employed 25–100 nM topoisomerase II and 5 nM (0.3 μg) negatively supercoiled pBR322 DNA in a total volume of 20 μL of cleavage buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM EDTA, and 2.5% glycerol) which contained 5 mM $MgCl_2$. Samples were incubated for 6 min at 30 °C. Cleavage products were trapped (Liu et al., 1983; Sander & Hsieh, 1983; Osheroff & Zechiedrich, 1987) by the addition of 2 μL of 10% SDS. One microliter of 250 mM EDTA and 2 μL of a 0.8 mg/mL solution of proteinase K were added, and samples were incubated at 37 °C for 30 min to digest the topoisomerase II. Final products were mixed with 2.5 μL of loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanole FF, and 10 mM Tris-HCl, pH 7.9), heated at 70 °C for 1 min, and subjected to electrophoresis in 1% agarose (MCB) gels in 40 mM Tris–acetate and 2 mM EDTA at 4 V/cm. Following electrophoresis, DNA bands were stained in a 1 μg/mL ethidium bromide solution and visualized by transillumination with ultraviolet light (300 nm). The bands were photographed through Kodak 24A and 12 filters using Polaroid type 665 positive/negative film. The amount of DNA was quantitated by scanning negatives with a Biomed Instruments Model SL-504-XL scanning densitometer. Under the described conditions, the intensity of the negative was directly proportional to the amount of DNA present.

A range of 0–60 μM drug was used to examine the effect of *m*-AMSA on the topoisomerase II mediated DNA cleavage/religation equilibrium. Control samples always contained an amount of DMSO equivalent to that in the drug-containing sample. Over the concentration range employed, DMSO had no effect on topoisomerase II activity. Reversal of topoisomerase II mediated DNA cleavage (in magnesium-containing reactions) was accomplished by the addition of NaCl (0.5 M final concentration) or EDTA (10 mM final concentration) prior to the addition of SDS.

Religation of Cleaved DNA by Topoisomerase II. To study religation uncoupled from the forward cleavage reaction, the assay of Osheroff and Zechiedrich (1987) was employed; 25–100 nM topoisomerase II and 5 nM negatively supercoiled pBR322 plasmid DNA (final concentration) were incubated at 30 °C for 6 min in 20 μL of cleavage buffer that contained 5 mM $CaCl_2$ in place of 5 mM $MgCl_2$. Kinetically competent topoisomerase II–DNA cleavage complexes were trapped by the addition of 0.8 μL of 250 mM EDTA. NaCl (0.6 μL of a 5 M solution) was added to prevent recleavage of the DNA during the religation assay (Liu et al., 1983; Osheroff & Zechiedrich, 1987), and samples were placed on ice to slow reaction rates. Religation was initiated by addition of cold $MgCl_2$ (8.5 mM final concentration). SDS (2 μL of a 10% solution) was added to terminate religation at several time points up to 30 s. Samples were digested with proteinase K and analyzed by electrophoresis as described above. In experiments which examined the effects of *m*-AMSA, 60 μM drug was added along with the topoisomerase II and the DNA during the initial cleavage incubation (i.e., prior to the addition of EDTA). Again, control samples contained an appropriate amount of DMSO and religation rates were not affected by its addition.

Results

Effect of m-AMSA on the DNA Cleavage/Religation Equilibrium of Drosophila Topoisomerase II. The effect of *m*-AMSA on the DNA cleavage/religation equilibrium of *D. melanogaster* topoisomerase II was studied by including 0–60 μM drug in reaction mixtures (Figure 1). In order to simplify the interpretation of results, all assays were carried out in the absence of ATP. Thus, reactions presented below represent the enzyme's DNA cleavage and religation events which take place prior to its DNA strand passage reaction (Hsieh & Brutlag, 1980; Osheroff et al., 1983; Osheroff, 1989a).

Over the concentration range employed, *m*-AMSA had a pronounced effect on the DNA cleavage/religation equilibrium

[1] Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

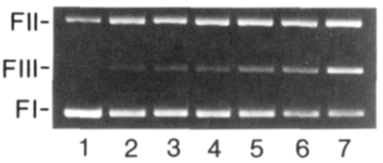

FIGURE 1: Effect of *m*-AMSA on the DNA cleavage/religation equilibrium of *D. melanogaster* topoisomerase II. Assays were carried out as described under Experimental Procedures. Lane 1, DNA standard; lane 2, DNA cleavage carried out in the absence of drug; lane 3, DNA cleavage carried out in the absence of drug but in the presence of 0.6% DMSO (the level of solvent present in drug-containing reactions); lanes 4–7, DNA cleavage carried out in the presence of 2.4, 12, 24, and 60 μM *m*-AMSA, respectively. The positions of fully supercoiled DNA (form I, FI), nicked circular plasmid molecules (form II, FII), and linear molecules (form III, FIII) are indicated.

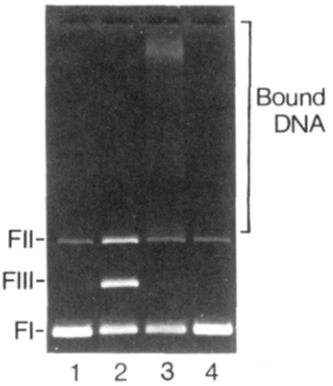

FIGURE 2: Effects of *m*-AMSA are mediated by topoisomerase II. Assays were carried out in the presence of 60 μM *m*-AMSA as described under Experimental Procedures. Lane 1, DNA standard; lane 2, DNA cleavage products which were digested with proteinase K; lane 3, DNA cleavage products which were not digested with proteinase K; lane 4, DNA cleavage which was carried out in the absence of topoisomerase II. The position of topoisomerase II bound DNA is indicated in addition to those of form I, II, and III molecules.

of the *Drosophila* enzyme. In the presence of 60 μM drug, topoisomerase II mediated double-stranded DNA cleavage increased ~5-fold (Figure 1). This stimulation of DNA breakage is similar to that previously reported for mammalian systems (Nelson et al., 1984; Pommier et al., 1984; Tewey et al., 1984).

When the concentration of *m*-AMSA was increased beyond the range shown in Figure 1, levels of DNA breakage mediated by *Drosophila* topoisomerase II were further enhanced. However, when the drug exceeded 60 μM, the presence of multiple enzyme–DNA cleavage complexes per plasmid molecule became apparent (not shown). This complicated the analysis of drug action by converting a significant portion of the DNA substrate to lengths smaller than unit size. Therefore, 60 μM *m*-AMSA was employed for all the experiments described below.

The effects of *m*-AMSA on DNA were mediated solely by topoisomerase II (Figure 2). In the absence of enzyme, incubation of 60 μM drug with DNA produced no nucleic acid cleavage (lane 4). Moreover, all of the linear (i.e., doubly cut) and nicked (i.e., singly cut) DNAs generated in the presence of drug and topoisomerase II were covalently attached to the enzyme (compare lanes 2 and 3). In order to release cleaved DNAs, topoisomerase II had to be digested with proteinase K. The covalent linkage of enzyme to cleaved DNA is a hallmark of the topoisomerase II mediated reaction (Liu et al., 1983; Sander & Hsieh, 1983; Osheroff & Zechiedrich, 1987).

Reversibility of DNA Breaks Formed by Topoisomerase II in the Presence of m-AMSA. The DNA cleavage reaction

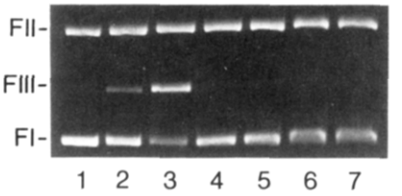

FIGURE 3: EDTA and salt reversibility of DNA breaks mediated by topoisomerase II. Assays were carried out as described under Experimental Procedures in the absence (lanes 2, 4, and 6) or the presence (lanes 3, 5, and 7) of 60 μM *m*-AMSA. Lane 1, DNA standard; lanes 2 and 3, SDS was added prior to EDTA; lanes 4 and 5, EDTA (10 mM final concentration) was added prior to SDS; lanes 6 and 7, NaCl (0.5 M final concentration) was added prior to SDS.

of topoisomerase II can be readily reversed if EDTA (in magnesium-containing reactions) (Sander & Hsieh, 1983; Osheroff & Zechiedrich, 1987) or salt (Liu et al., 1983; Osheroff & Zechiedrich, 1987) is added to assay mixtures prior to the addition of SDS. This demonstrates that topoisomerase II which is sequestered within the covalent enzyme–DNA cleavage complex must be kinetically competent.

Previous studies indicate that the *m*-AMSA-induced DNA cleavage generated by mammalian topoisomerase II is salt reversible (Pommier et al., 1984; Tewey et al., 1984). The following experiments were carried out to examine the reversibility of DNA cleavage mediated by the *Drosophila* enzyme in the presence of the drug (Figure 3). First, 10 mM EDTA was added to reaction mixtures after the DNA cleavage/religation equilibrium had been established, but before SDS treatment (lanes 4 and 5). Second, the experiment was repeated, but 0.5 M salt was added in place of the 10 mM EDTA (lanes 6 and 7). In both cases, levels of reversibility observed in the presence of 60 μM *m*-AMSA were comparable to those found in the absence of drug. Two conclusions can be drawn from these results. (1) The *Drosophila* topoisomerase II–DNA cleavage complex formed in the presence of *m*-AMSA is both viable and kinetically competent. (2) *m*-AMSA stabilizes, but does not permanently trap, the enzyme–DNA cleavage complex. Thus, in kinetic terms, the drug must act either by enhancing the forward rate of DNA cleavage or by inhibiting the enzyme's ability to religate its cleaved DNA intermediate (or both).

Inhibition of Topoisomerase II Mediated DNA Religation by m-AMSA. Under conditions where the nonintercalative drug etoposide (Ross et al., 1984; Chow et al., 1988) enhances DNA breakage by *Drosophila* topoisomerase II ~5.5-fold, it inhibits the enzyme's ability to religate cleaved DNAs by ~3-fold (Osheroff, 1989b). Thus, it was concluded that etoposide stabilized the DNA cleavage complex of topoisomerase II (at least in part) by inhibiting its religation reaction. In order to establish whether the intercalative drug *m*-AMSA stabilizes the DNA cleavage complex by a similar mechanism, the effect of 60 μM drug on the enzyme's ability to religate cleaved DNA was determined. To this end, the DNA religation assay of Osheroff and Zechiedrich (1987) was employed. This assay, which is detailed under Experimental Procedures, takes advantage of the fact that calcium can be used to trap the topoisomerase II–DNA cleavage complex in an active form.

Previous studies on the topoisomerase II mediated DNA religation reaction indicate that the enzyme rejoins double-stranded breaks one strand at a time (Zechiedrich et al., 1989; Andersen et al., 1989). The first religation event (i.e., the conversion of linear to nicked DNA) can be quantitated by monitoring the loss of linear (form III) DNA. Likewise, the second event (i.e., the conversion of nicked to supercoiled

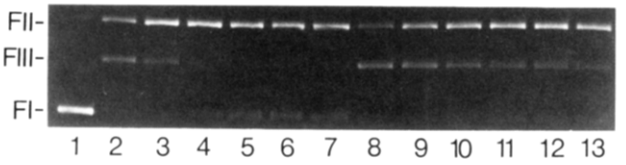

FIGURE 4: Effect of *m*-AMSA on the ability of topoisomerase II to religate cleaved DNA. Assays were carried out as described under Experimental Procedures in the absence (lanes 2–7) or the presence (lanes 8–13) of 60 μM *m*-AMSA. Lane 1, DNA standard; lanes 2–7 and 8–13, religation times of 0, 5, 10, 15, 20, and 30 s, respectively.

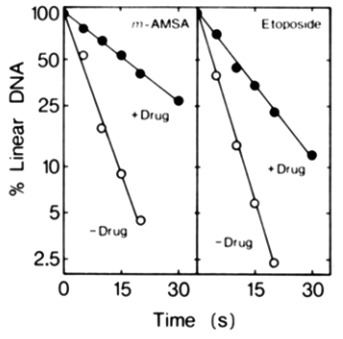

FIGURE 5: Comparison between the effects of 60 μM *m*-AMSA (left panel) and 100 μM etoposide (right panel) on the DNA religation reaction of topoisomerase II. A semilogarithmic plot of percent DNA versus time is shown. The religation of the first DNA strand (i.e., the conversion of linear DNA to nicked circular molecules) is displayed. Results are plotted as the loss of linear DNA in the absence (open circles) or the presence (closed circles) of drug. The etoposide results were taken from Osheroff (1989b).

DNA) can be followed by monitoring the reappearance of supercoiled (form I) plasmid. The results of a typical religation assay are shown in Figure 4. In this experiment, enzyme concentrations were adjusted so that the initial levels of DNA breakage observed at time zero were similar in the absence (lane 2) or presence (lane 8) of *m*-AMSA. As determined by either the loss of linear DNA or the increase in supercoiled plasmid, 60 μM *m*-AMSA clearly inhibits the ability of topoisomerase II to religate its cleaved DNA intermediate.

The inhibitory properties of *m*-AMSA were compared to those previously established for etoposide (Osheroff, 1989b) by quantitating the effect of drug on religation of the first DNA strand. Results of a typical religation assay are presented as semilogarithmic plots of percent linear DNA versus time (Segel, 1975; Fersht, 1985) in Figure 5 (left panel). Previous results with etoposide are presented in the right panel for comparison. *m*-AMSA inhibited the rate of religation ~3.5-fold. As calculated from the plots shown in Figure 5, the drug lowered the apparent first-order rate constant for the reaction from 0.17 to 0.05 s^{-1}. In two additional and independent experiments, *m*-AMSA was found to inhibit DNA religation 3.6- and 4.0-fold, respectively (not shown).

It should be noted that in the above experiments, *m*-AMSA was present at the time the initial DNA cleavage/religation equilibrium was established. If the drug was excluded from assay mixtures until after the topoisomerase II–DNA cleavage complex was trapped with EDTA (Osheroff & Zechiedrich, 1987; Gale & Osheroff, 1989), its effect on religation rates was greatly diminished (not shown). Thus, in order to inhibit the enzyme's religation ability, it appears that *m*-AMSA needs to be present at the time of the initial cleavage event.

Discussion

Topoisomerase II is the primary cellular target for a number of antineoplastic drugs which are relevant to the treatment of human cancers (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). Although the mechanism by which these drugs kill cells has not yet been established, their clinical efficacies correlate with their abilities to stabilize the enzyme's DNA cleavage complex (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). Results of the present study indicate that the topoisomerase II targeted drug *m*-AMSA stabilizes this complex primarily by inhibiting the enzyme's ability to religate its cleaved DNA intermediate.

Different classes of topoisomerase II targeted antineoplastic drugs display very different DNA binding characteristics (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). Thus, it has been questioned whether nonintercalative drugs such as etoposide and intercalative drugs such as *m*-AMSA stabilize the enzyme's cleavage complex via a single common mechanism or rather produce the same net result via two different pathways (Zwelling, 1985; Glisson & Ross, 1987; Lui, 1989). A comparison between this study and a recent report on etoposide (Osheroff, 1989b) argues for a common mechanism of drug action (at least at the level of enzyme kinetics). First, both etoposide and *m*-AMSA interfered with topoisomerase II mediated DNA religation. Second, under conditions where etoposide and *m*-AMSA enhanced enzyme-mediated DNA breakage ~5.5- and ~5-fold respectively, the drugs inhibited the enzyme's ability to rejoin cleaved DNAs ~3- and ~3.5-fold respectively. Thus, both drugs show a similar ability to inhibit religation relative to their stabilization of the enzyme's cleavage complex. Third, both etoposide and *m*-AMSA needed to be present at the time of DNA cleavage in order to affect topoisomerase II mediated DNA religation. This latter finding makes it likely that both antineoplastic agents exert their actions within the ternary enzyme–DNA complex. This is supported by the preliminary finding that etoposide may be able to bind topoisomerase II in addition to DNA (Osheroff & Gale, 1988) and studies which demonstrate that the efficacies of topoisomerase II targeted drugs do not correlate with their abilities to bind DNA (Wilson et al., 1981; Chow et al., 1988).

It should be noted that the efficacy of *m*-AMSA relative to that of etoposide (i.e., the level of DNA breakage at any given drug concentration) was somewhat lower with *Drosophila* topoisomerase II than previously described with mammalian systems (Ross et al., 1984; Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). This may reflect subtle differences between the catalytic properties of the *Drosophila* and mammalian enzymes or the fact that the present study employed purified topoisomerase II and DNA in the absence of other proteinaceous and chemical components usually found in the cell nucleus. Finally, since assays were carried out in the absence of ATP [the high-energy cofactor which is required for enzyme-mediated DNA strand passage (Vosberg, 1985; Wang, 1985; Osheroff, 1989a)], the current work describes only the effects of *m*-AMSA on the pre-strand passage DNA cleavage/religation equilibrium of topoisomerase II. Studies examining the effects of *m*-AMSA on the enzyme's post-strand passage DNA cleavage/religation equilibrium (Osheroff, 1986) are currently underway in the laboratory and may be required to fully describe the mechanism by which antineoplastic drugs alter the biological functions of topoisomerase II.

Understanding the mechanism by which a drug exerts its biological actions is imperative for rational drug design. It now appears that two structurally disparate topoisomerase II targeted antineoplastic drugs share a common kinetic mode of action. On the basis of this finding, it seems likely that most,

if not all, chemotherapeutic drugs which act through topoisomerase II will display a similar ability to interfere with the enzyme's religation event.

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Lead-Catalyzed Cleavage of Yeast tRNA^{Phe} Mutants†

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ABSTRACT: Yeast tRNA^{Phe} lacking modified nucleotides undergoes lead-catalyzed cleavage between nucleotides U17 and G18 at a rate very similar to that of its fully modified counterpart. The rates of cleavage for 28 tRNA^{Phe} mutants were determined to define the structural requirements of this reaction. The cleavage rate was found to be very dependent on the identity and correct positioning of the two lead-coordinating pyrimidines defined by X-ray crystallography. Nucleotide changes that disrupted the tertiary interactions of tRNA^{Phe} reduced the rate of cleavage even when they were distant from the lead binding pocket. However, nucleotide changes designed to maintain tertiary interactions showed normal rates of cleavage, thereby making the reaction a useful probe for tRNA^{Phe} structure. Certain mutants resulted in the enhancement of cleavage at a "cryptic" site at C48. The sequences of *Escherichia coli* tRNA^{Phe} and yeast tRNA^{Arg} were altered such that they acquired the ability to cleave at U17, confirming our understanding of the structural requirements for cleavage. This mutagenic analysis of the lead cleavage domain provides a useful guide for similar analysis of autocatalytic self-cleavage reactions.

Certain ions, including Pb^{2+}, Zn^{2+}, and Eu^{3+}, are capable of cleaving purified tRNAs at precise locations to give 2′,3′ cyclic phosphate and 5′ hydroxyl termini (Rordorf et al., 1976; Werner et al., 1976; Ciesiolka et al., 1986). The best studied example of this phenomena is the lead-induced cleavage of yeast tRNA^{Phe} between residues D17 and G18 (Dirheimer et al., 1972; Werner et al., 1976; Krzyzosiak et al., 1988). X-ray diffraction studies on the cleaved and uncleaved tRNA^{Phe}–lead complex implicated one of the three tightly bound lead ions in the cleavage reaction (Brown et al., 1983, 1985; Ruben & Sundaralingam, 1983; Sundaralingam et al., 1984). This lead ion, termed Pb(1), is precisely coordinated in a pocket formed

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