labeled with ^{32}P and the second is a phosphorothioate. The sequence of the tetramer from the control RNA was confirmed by nearest neighbor analysis. Each tetranucleotide was digested with snake venom phosphodiesterase (SVPDE), which has a 1700-fold greater activity on R_p isomers (*8*, *13*), and the products were separated by gel electrophoresis (Fig. 2). The control RNA (R_p isomer) was digested to mononucleotides, with less than 1 percent of the ^{32}P in the dinucleotide region. The ligated product RNA was only digested to the labeled dinucleotide pUpA. Quantitation of the gel on a β-scanner (Betagen) showed that less than 2 percent of the radioactivity was in mononucleotides. The inability of the phosphodiesterase to cleave the dinucleotide to mononucleotides indicates that the phosphorothioate product is S_p, and therefore that the ribozyme reaction proceeds with inversion of configuration. The experiment was repeated with (α-^{35}S)-labeled rATP to label the ligator and control RNA's, and the same result was obtained: the phosphorothioate in the ribozyme-generated RNA was resistant to phosphodiesterase cleavage, while the control RNA was digested to mononucleotides.

Replacing the pro-*R* oxygen of the reactive phosphate with sulfur decreases the rate of reactions by a factor of about 1000. One possible explanation for this effect is that the larger sulfur interferes with the coordination of a Mg^{2+} ion that is normally bound to the two phosphate oxygens, where it functions either in substrate binding, in stabilizing the transition state, or in charge neutralization to facilitate the nucleophilic attack. This rate decrease notwithstanding, it is clear from a number of cases where the stereochemical course of enzymic reactions has been studied both by the use of ^{16}O, ^{17}O, or ^{18}O phosphodiester groups and by the use of a phosphorothioester, that the elemental substitution does not affect the stereochemical consequence at phosphorus (*8*, *14*).

Any odd number of nucleophilic substitution reactions at phosphorus can result in inversion of configuration of the phosphorothioate (*7*, *8*). In the simplest scheme, a single displacement reaction occurs in which the enzyme catalyzes the nucleophilic attack of the 3′ hydroxyl of the primer on the first internal phosphate of the ligator, resulting in product formation. The 5′-guanosine residue of the ligator is released. In the transition state of such a reaction the phosphorus has trigonal bipyramidal geometry, with incoming and leaving groups in the axial positions.

This mechanism implies an in-line orientation of the incoming and leaving hydroxyl groups. It seems likely that this mechanism will apply to all of the phosphotransfer reactions catalyzed by the ribozyme. If so, the guanosine that attacks the P1 stem in the first step of self-splicing must be oriented by the enzyme so that its O3′ is opposite the O3′ of the leaving exon. This is an important constraint in attempts to model the active site of the enzyme.

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Identification of the Fusion Peptide of Primate Immunodeficiency Viruses

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Membrane fusion induced by the envelope glycoproteins of human and simian immunodeficiency viruses (HIV and SIV_{mac}) is a necessary step for the infection of CD4 cells and for the formation of syncytia after infection. Identification of the region in these molecules that mediates the fusion events is important for understanding and possibly interfering with HIV/SIV_{mac} infection and pathogenesis. Amino acid substitutions were made in the 15 NH_2-terminal residues of the SIV_{mac} gp32 transmembrane glycoprotein, and the mutants were expressed in recombinant vaccinia viruses, which were then used to infect CD4-expressing T cell lines. Mutations that increased the overall hydrophobicity of the gp32 NH_2-terminus increased the ability of the viral envelope to induce syncytia formation, whereas introduction of polar or charged amino acids in the same region abolished the fusogenic function of the viral envelope. Hydrophobicity in the NH_2-terminal region of gp32 may therefore be an important correlate of viral virulence in vivo and could perhaps be exploited to generate a more effective animal model for the study of acquired immunodeficiency syndrome.

THE ENVELOPE GLYCOPROTEINS OF SIV_{mac} (gp120 and gp32) are derived from a gp160 precursor through endoproteolytic cleavage and contain all determinants necessary for host cell infection and syncytium formation (*1*). The gp32, like the gp41 of HIV-1 (*2*), is the transmembrane glycoprotein (*3*) that is expressed on the surface of infected cells and incorporated in the viral membrane; gp32 anchors gp120, which contains the binding site for the CD4 antigen (*4*, *5*). The smaller size of the SIV_{mac} transmembrane glycoprotein (gp32 as opposed to HIV-1 gp41) is due to the presence of a premature termination codon in the SIV_{mac} *env* gene (*6*); some data indicate that the region after the termination codon is expressed in vivo in SIV_{mac}-infected animals (*7*).

Several studies indicate that the hydrophobic NH_2-terminus of the transmembrane glycoprotein is primarily responsible for the membrane fusion events involved with HIV and SIV_{mac} infection and with syncytium formation. This region has amino acid sequence similarity to the fusion peptides of ortho- and paramyxoviruses (*8*), and it has been shown, as for other enveloped viruses, that the cleavage of the *env* precursor resulting in exposure of the gp41 NH_2-terminus is a necessary event for HIV infectivity (*9*). Furthermore, insertion mutagenesis performed on this region of the HIV-1 gp41 (inserting four to six amino acids after position 6 of the gp41) abolishes the capacity of the envelope glycoproteins to induce syncytia in a heterologous expression system (*4*).

The fusion peptides of ortho- and para-

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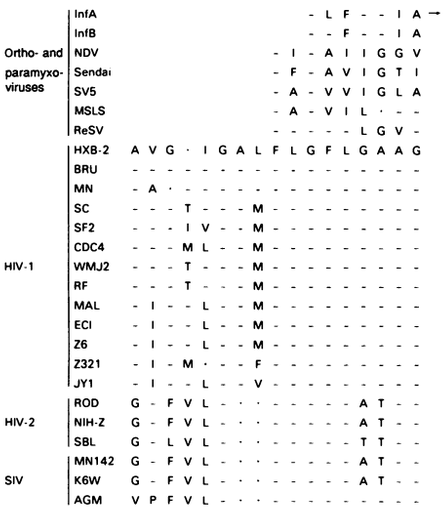

Fig. 1. Sequence comparison of the fusion peptides of ortho- and paramyxoviruses with the NH_2-termini of transmembrane *env* glycoproteins. Amino acids are in the single letter code (*12*). Data for influenza A and B (InfA and InfB), Newcastle disease virus (NDV), Sendai virus and Sendai virus 5 (SV5), measles virus (MSLS), and respiratory syncytia virus (ReSV) were taken from (*13*), data for HIV-1, HIV-2, and SIV_{mac} strains from (*14*), except for HIV-$2_{SBL/ISY}$ (*15*) and SIV_{AGM} (*16*).

myxoviruses and the sequences of the NH_2-terminal regions of the transmembrane glycoproteins of the different HIV and SIV_{mac} isolates (Fig. 1) appear to contain a phenylalanine-*X*-glycine motif, where *X* is generally a leucine in the immunodeficiency viruses. Although this motif is not NH_2-terminal in the HIV and SIV_{mac} transmembrane glycoproteins, it is found once (in SIV_{mac} and HIV-2) or twice (in HIV-1) within the first 10 to 15 amino acids. Furthermore, glycine residues appear to be conserved at regularly spaced intervals in most of the sequences listed in Fig. 1. Because glycines have an extremely short side chain (−H), this repetition may have structural implications. In fact, when glycine residues are not conserved in all sequences, they are often replaced by alanine residues, which also have very short side chains ($-CH_3$).

To determine if the NH_2-terminus of gp32 does constitute the fusion peptide of SIV_{mac}, we made a series of nucleotide changes in a DNA fragment coding for the NH_2-terminus of the SIV_{mac} gp32 (Fig. 2). The mutated DNA sequences were inserted into the vaccinia virus pSC11 vector (*9*) containing the SIV_{mac} *env* gene under control of the vaccinia promoter (construct pA194 or SE). Recombinant vaccinia viruses expressing the mutated SIV_{mac} *env* genes were generated by homologous recombination (*10, 11*) and used to infect the human neoplastic $CD4^+$ T cell lines CEM-SS and H9. Wild-type SIV_{mac} *env* glycoproteins expressed in this manner generated syncytia in these cells (Fig. 3A), and we studied the effect of the amino acid changes on this capacity to induce membrane fusion. These changes could be divided into two groups: mutations that increased the hydrophobicity (Fig. 3B) and mutations that introduced polar or charged residues, thereby decreasing the overall hydrophobicity (Fig. 3C).

Two of the mutations in the SIV_{mac} *env* gene, SEΔ1A and SEΔ9A, exchanged a glycine for a more hydrophobic alanine residue (Fig. 2). Both of these mutants induced more and larger syncytia than wild type (Fig. 3, A and B), showing that the gp32 NH_2-terminus is responsible for the fusogenic capacity of the SIV_{mac} *env* gene products and that an increase in hydrophobicity of this region can enhance syncytia formation. Syncytia formation was enhanced even in mutant SEΔ9A, where the glycine of the central Phe-Leu-Gly motif was replaced. SEΔ1A has an NH_2-terminal alanine, as do all HIV-1 isolates (Fig. 1). Because HIV-1, in general, seems to be more cytopathic in vitro than SIV_{mac}, one might conclude that the primary structure of the fusion peptides of these viruses is correlated with this higher cytopathicity in vitro. In mutant SEΔ3L, phenylalanine at position 3 was replaced with a leucine, as in HIV-$2_{SBL/ISY}$, and there was a slight increase in syncytium

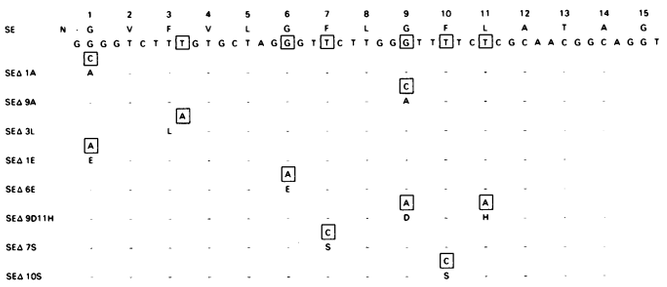

Fig. 2. Amino acid changes introduced in the NH_2-terminus of the SIV_{mac} gp32. A Cla I–Nhe I fragment from the SS35 subclone of STLV-III/K6W (*17*) was cloned into M13mp19 and subjected to mutagenesis according to Kunkel (*18*) with the Bio-Rad mutagen kit reagents. The oligonucleotides used for mutagenesis were (5′→3′) GCACAAAGACCGCTCTTTATTTCTTGAGG (SEΔ1A), GCACAAAGACCTCTCTTTATTTCTTGAGG (SEΔ1E), CCCTAGCACTAAGACCCC (SEΔ3L), CCCAAGAACTCTAGCAC (SEΔ6E), CGTTGCGTGAAAATCCAAGAACCC (SEΔ9D11H), CCCAAGGACCCTAGCAC (SEΔ7S), GCGAGAAAAGCCAAGAACCC (SEΔ9A), and GCCGTTGCGAGAGAACCCAA (SEΔ10S). The mutated Cla I–Nhe I fragments were recloned into SS35. Subsequently, the Ava III–Nhe I fragments from SS35 were cloned into the vaccinia-SE construct containing the SIV_{mac} *env* gene from position 1 to the premature termination codon (734 amino acids).

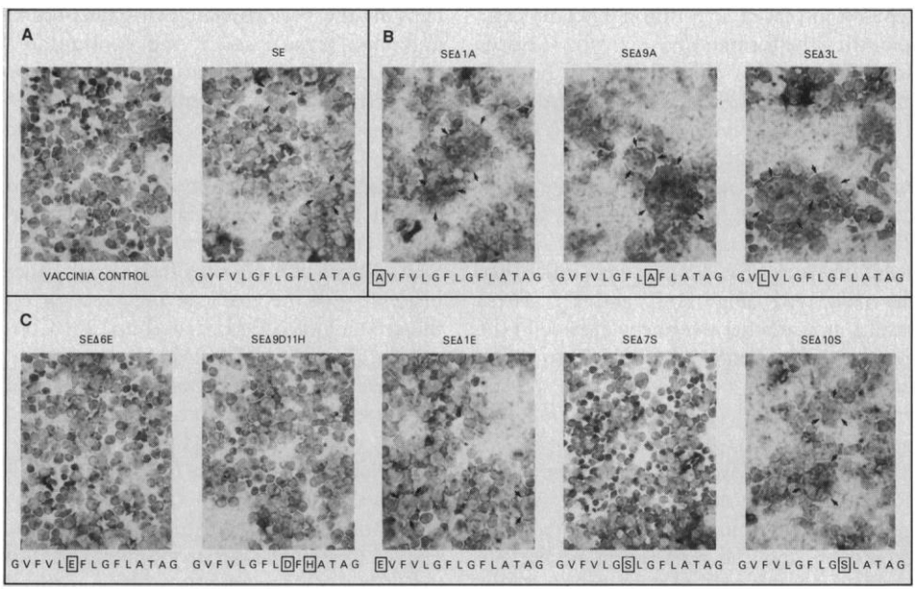

Fig. 3. CEM-SS cells infected with recombinant vaccinia viruses containing SIV_{mac} *env* mutations. (**A**) Wild-type SIV_{mac} *env* glycoproteins. (**B**) Mutations that increased hydrophobicity. (**C**) Mutations that decreased hydrophobicity. CEM cells (1×10^5) were infected with vaccinia virus in flat-bottom 96-well microtiter trays at a multiplicity of infection (MOI) of 100 in 200 μl of RPMI 1640 medium supplemented with 10% fetal bovine serum. After 16-hour (overnight) incubation the cells were resuspended and spun onto slides at 500 rpm for 5 min. The cells were stained for 10 min in Wright-Giemsa stain and rinsed in distilled water before photography with a Zeiss microscope (×123).

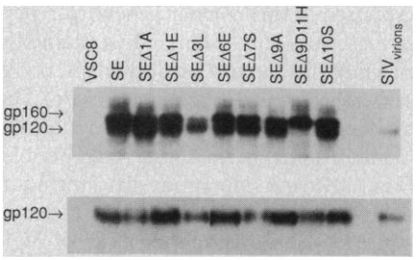

Fig. 4. Autoradiograph of protein immunoblot analysis of CEM-SS–infected cells with the vaccinia SIV_{mac} *env* constructs indicated above each lane. CEM-SS cells (1×10^6) were infected with recombinant vaccinia viruses at an MOI of 100. After 24 hours the cells were harvested and lysed with 0.5% NP-40. The protein content of each cellular lysate was calculated with the Bio-Rad protein assay with bovine serum albumin standards. Proteins (50 μg per lane) from each cellular lysate and disrupted SIV_{mac} virions, as a positive control, were separated by electrophoresis on a 7% polyacrylamide gel and transferred to a nitrocellulose filter (upper panel). The supernatants of the infected cells were concentrated by centrifugation, separated on a 7% polyacrylamide gel, and transferred to nitrocellulose filters (lower panel). The filters were reacted with a monkey serum (F-280) obtained from an experimentally inoculated animal (7), and iodinated Staphylococcus Protein A was used to detect immunocomplexes.

Table 1. Syncytium-inducing capabilities of the different SIV_{mac} *env* mutants.

Mutant	Fusion index	CEM-SS cells			H9 cells		
		%*	Size†	State‡	%*	Size†	State‡
SE	1	++	++	++	+	+	+++
SEΔ1A	6	+++++	+++++	+	−	+	+
SEΔ9A	6	+++++	+++++	+	−	−	+
SEΔ3L	1.5	++	+++	++	+	+	++
SEΔ1E	0.25	+	+	++	+	+	++
SEΔ6E	0.1	−	+	++	−	−	++
SEΔ9D11H	0	−	−	+++	−	−	+++
SEΔ7S	0.1	−	+	++	−	+	+++
SEΔ10S	1	++	++	+	+	+	+

*Symbols: −, <5% of cells affected; each + increases the percentage of affected cells by 5%. †Symbols: −, <3 cells per syncytium; +, 3 to 5 cells per syncytium; ++, 5 to 10 cells per syncytium; +++, 10 to 20 cells per syncytium; and +++++, >25 cells per syncytium. ‡Symbols: +, cells are fragile and expanded; membrane is not well defined; ++, mixture or intermediate stage; and +++, cells are healthy and round.

formation compared to wild type (Fig. 3, A and B).

Introduction of charged amino acids, except when placed at position 1, completely abolished the formation of syncytia (SEΔ6E and SEΔ9D11H) (Fig. 3C). The replacement of the NH_2-terminal glycine by a glutamic acid (SEΔ1E) had little effect (Fig. 3C). The exchange of phenylalanines (apolar) with serines (polar) had varying effects depending on the location. Mutant SEΔ7S did not induce any syncytia in CEM-SS cells, whereas SEΔ10S behaved much like the wild type (Fig. 3, A and C). These results indicate that the phenylalanine in the Phe-Leu-Gly motif (positions 7 to 9) is more important for the function of the fusion peptide than the phenylalanine at position 10.

Because the cleavage of the *env* precursor could be influenced by mutations in the gp32 NH_2-terminus, we analyzed the expression and the maturation of the *env* glycoproteins in the SIV_{mac} *env* mutants. We performed protein immunoblots on the total cellular proteins and supernatants of the cells infected with the vaccinia recombinant viruses. All the SIV_{mac} *env* mutants, with the exception of SEΔ9D11H, showed an amount of cell-associated gp120 comparable to that of the wild-type SE (Fig. 4, upper panel), which indicated adequate processing of the envelope precursor proteins in the various mutants. The protein immunoblot analysis of the concentrated supernatants from the infected cells showed a substantial amount of gp120 released into the medium (Fig. 4, lower panel) in all the SIV_{mac} *env* mutants. These data indicate that the increased ability of SEΔ1A and SEΔ9A to induce syncytia and the abolishment of this function in mutants SEΔ6E and SEΔ7S does not rely on alteration of either processing of the envelope precursor or on the amount of cell-associated gp120. Conversely, in mutant SEΔ9D11H, the abolishment of syncytia induction was correlated with the lack of detection of cell-associated gp120. Because we introduced two charged amino acids to generate this mutant, it is conceivable that such a change alters the ability of the mutated gp32 to anchor the gp120. One of the putative binding sites of the HIV-1 gp41 to gp120 has been mapped immediately downstream from this region (*4*).

Different cell lines reacted differently with the SIV_{mac} *env* mutants. Whereas SEΔ1A induced giant syncytia in CEM-SS cells, this mutant induced no syncytia in H9 cells. However, H9 cells seemed to show cytopathic effects (CPE), becoming permeabilized and ghost-like in appearance after infection and overnight incubation (Table 1). The same effect was observed in H9 cells infected with construct SEΔ9A and, in general, the fusogenic properties of the mutants in CEM-SS cells was reflected as CPE in H9 cells. Likewise, the least fusogenic mutants (in CEM-SS cells) had little or no effect on the H9 cells (Table 1; SEΔ6E, SEΔ9D11H, and SEΔ7S).

An increase in hydrophobicity increased syncytium formation, as in SEΔ1A and SEΔ9A. Although the mechanism through which the fusion peptide induces membrane fusion is unknown, insertion of this peptide into the membrane of the target cell may be a necessary step for infection or syncytium formation. Because increased hydrophobicity would enhance the potential for interaction with the cell membrane, this theory would explain the stronger fusion activity of mutants SEΔ1A and SEΔ9A.

The change of the NH_2-terminal SIV_{mac}- and HIV-2–specific glycine at position 1 to an alanine increased the CPE of the expressed *env* gene products, as did a similar change at position 9. The strong conservation of the NH_2-terminal alanine residue in HIV-1 and of the glycine residue at the homologous position in SIV_{mac} and HIV-2 may reflect the importance of this in the biology of these viruses. The glycine to alanine change increased the cytopathic potential of the SIV_{mac} *env* glycoproteins when expressed in vaccinia, and this change could, therefore, lead to the construction of a pathogenic molecular clone of SIV_{mac}. The availability of such a cloned virus could greatly enhance the use of animal systems as models for research on human acquired immunodeficiency syndrome.

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12. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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19. We thank P. Johnson for the SE (pA194) construct, P. Kanki for the SIV_{mac} serum, and R. C. Gallo and B. Moss for support. M.B. is a fellow of the Dutch Society for the Advancement of Pure Research (ZWO).

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Calicheamicin γ_1^I and DNA: Molecular Recognition Process Responsible for Site-Specificity

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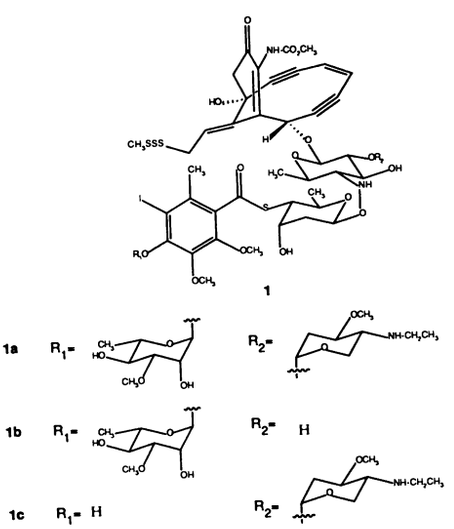
Calicheamicin γ_1^I is a recently discovered diyne-ene–containing antitumor antibiotic that cleaves DNA in a double-stranded fashion, a rarity among drugs, at specific sequences. It is proposed that the cutting specificity is due to a combination of the complementarity of the diyne-ene portion of the aglycone with DNA secondary structures and stabilization by association of the thiobenzoate-carbohydrate tail with the minor groove.

AN IMPORTANT AND INTRIGUING aspect of the phenomenally potent antitumor agent calicheamicin γ_1^I (structure **1a**) (*1, 2*) is its interaction in vitro with DNA in which the drug makes double-stranded cuts at precise sequences (*3*). The DNA cleaving properties are believed to be initiated by the formation of a 1,4-diyl (*p*-benzyne) intermediate (structure **2**). The unexpected site-specificity of this chemistry is far from obvious, since a similar and equally potent compound, esperamicin (structure **3**) (*4, 5*), does not exhibit any strong sequence-specificity (*6*). Experimental evidence, in conjunction with molecular modeling studies reported here, provides insight into the calicheamicin-DNA encounter and may explain the distinctive sequence-specificity of such a small compound.



In order to determine the near-neighbor effect on the preferred sites TCCT/AGGA (*3*), a series of synthetic dodecamers were prepared with the tetramer sequence TCCT located at both the 5′ and 3′ ends as well as in the middle of the oligomers. Only the dodecamers having the TCCT sequence present in the middle and at the 3′ end were cut by **1a**, whereas the oligomer with the TCCT site placed at the 5′ end was not cut under these conditions (Fig. 1). The addition of a hanging sequence on either strand of the uncut dodecamer ending with 5′TCCT/3′AGGA does not facilitate or amplify cleavage (Fig. 1). This result indicates that calicheamicin γ_1^I anchors itself on the DNA from the 5′ side of the TCCT sequence (3′ side of the complementary AGGA sequence) and that **1a** requires double-stranded helical DNA in close proximity to the target site in a manner reminiscent of a restriction endonuclease.

Only the TCCT/AGGA site was subject to drug-induced cleavage and therefore this oligonucleotide-based system mimics the calicheamicin-polynucleotide restriction fragment studies (*3*). To gain further insight as

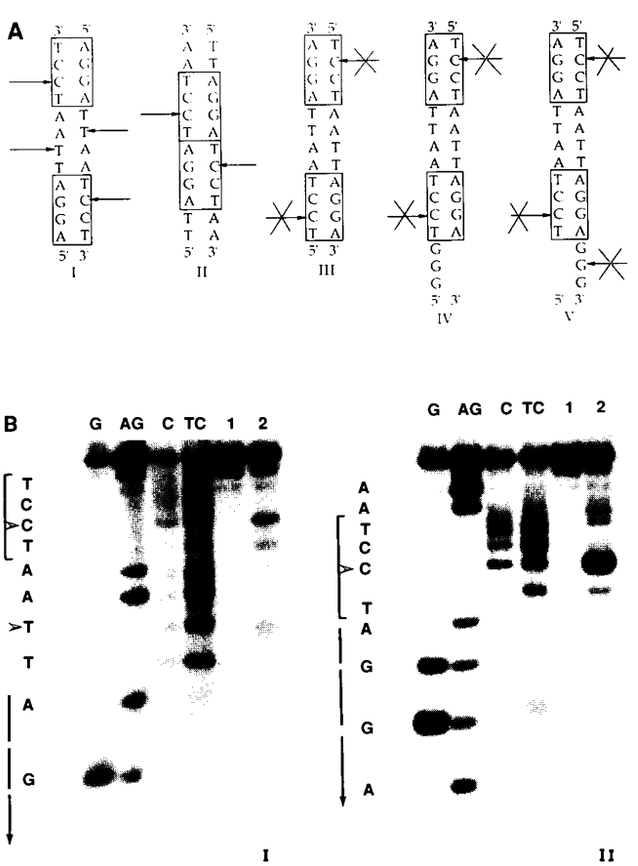


Fig. 1. (**A**) Calicheamicin γ_1^I double-strand cleavage sites on several synthetic oligomers with TCCT/AGGA sequences placed at different positions. The arrows indicate the calicheamicin γ_1^I sites of attack. (**B**) Autoradiograms of high-resolution denaturing gels of calicheamicin γ_1^I cleavage of 5′ end-labeled dodecamers I and II. Reaction conditions were 10:90 ethanol:50 m*M* tris-HCl, *p*H 7.5, carrier calf thymus DNA at 50 μg/ml [20*M* excess (in base pairs) to the drug], calicheamicin γ_1^I at 5 μg/ml, 10 m*M* β-mercaptoethanol, and end-labeled oligomer (~6000 cpm) in a total volume of 10 μl. Reactions were run for 2 hours at 4°C, then lyophilized. Cleavage products were analyzed on a 20% polyacrylamide sequencing gel at 2000 volts for 75 min. G, AG, C, TC are Maxam-Gilbert chemical sequencing lanes. Lanes 1, DNA controls; lanes 2, reactions with calicheamicin γ_1^I.

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