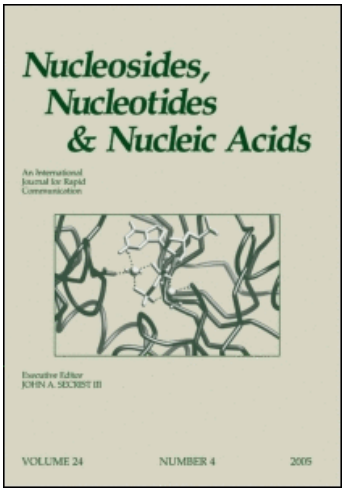

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RARE EARTH METAL IONS FOR DNA HYDROLYSES AND THEIR USE TO ARTIFICIAL NUCLEASE [1]

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ABSTRACT

Phosphodiester linkages in linear DNAs are efficiently hydrolyzed by rare earth metal salts. The activities of $CeCl_3$ and $Ce(NH_4)_2(NO_3)_6$ are especially large. Artificial hydrolytic nuclease for highly selective scission of DNA has been prepared by the attachment of Ce(IV) ion to a DNA oligomer as a sequence recognizing moiety.

Recently significant interest has been focused onto the molecular design of artificial nucleases. Selective cleavage of DNA by oxidative pathways has been successfully achieved.[2-5] However, DNA scission via hydrolysis of the phosphodiester linkages has not been successful yet;[6-8] the linkages are too stable to be hydrolyzed under normal conditions (the half-life at pH 7, 25°C has been estimated to be 200 million years).[9] The hydrolytic scission is advantageous, since no diffusible species is involved and thus clear-cut scission is plausible. In addition, the resultant DNA fragments can be enzymatically religated and be used for various purposes when necessary.

Barton *et al* reported that a supercoiled plasmid DNA is cleaved, partially via a hydrolytic pathway, by the combination of a ruthenium complex and a metal ion.[10] However, there was no report on the non-enzymatic hydrolysis of linear DNAs under physiological conditions.[11]

This paper is dedicated to Prof. Morio Ikehara, who is one of the most important pioneers in nucleoside and nucleotide chemistry, on the occasion of his 70th birthday.

Quite recently,[12,13] we have succeeded in the first non-enzymatic hydrolyses of linear DNAs by the use of lanthanide metal ions as catalysts. Lanthanide metal complexes of macrocyclic ligands were also active for the DNA hydrolysis.[14,15]

We report here the results of a detailed analysis on the DNA hydrolysis by rare earth metal ions. Catalytic activities for the scission as well as the base-specificities are clarified. A reaction mechanism is proposed on the basis of the kinetic analysis. Furthermore, an artificial nuclease which cleaves DNA selectively at the target site is prepared by the attachment of a rare earth metal ion to a DNA oligomer as a sequence-recognizing moiety.

EXPERIMENTAL SECTION

Materials: Thymidylyl(3'-5')thymidine (TpT) and 2'-deoxyadenylyl(3'-5')-2'-deoxyadenosine (d(ApA)) were purchased from Sigma. Rare earth metal salts were obtained from Soekawa (except for $LaCl_3$ from Nacalai). DNA oligomers were prepared by an automated synthesizer and were ^{32}P-labeled either at the 5'-end (by adenosine 5'-[γ-^{32}P]triphosphate) or at the 3'-end (by dideoxyadenosine 5'-[α-^{32}P]triphosphate). All the buffers were sterilized immediately before use. The greatest caution was paid to avoid the contamination of natural nucleases.

Hydrolysis of DNA Oligomers and Dinucleotides: Hydrolysis of ^{32}P-labeled DNA oligomers (10^{-6} *M*) was carried out in 50 *mM* Tris buffer and was followed by electrophoresis using denaturing polyacrylamide gel. The patterns were analyzed by a densitometer.

The dinucleotide hydrolysis in Hepes buffers was analyzed by a reversed-phase HPLC (Merck LiChrosphere RP-18(e) ODS column; water/acetonitrile = 92/8 (v/v)). The initial concentration of the dinucleotide was 10^{-4} *M*. Assignment of the signals was achieved by coinjection of the authentic samples.

Determination of Complex Formation Constant between Dinucleotide and Rare Earth Metal Ion: The complex formation constant K was determined by ^1H-NMR spectroscopy. The chemical shift changes ($\Delta\delta_{obs}$) of TpT, observed on the addition of rare earth metal ion to aqueous solution of TpT, were analyzed according to eq. 1.[16]

$$\Delta\delta_{obs} = [TpT]_0 + [Ln]_0 + 1/K - \{([TpT]_0 + [Ln]_0 + 1/K)^2 - 4[TpT]_0[Ln]_0\}^{1/2}/(2[TpT]_0/\Delta\delta_0) \quad (1)$$

The charged concentration of rare earth metal ion ($[Ln]_0$) was varied from 10^{-3} to $5x10^{-2}$ *M*, whereas $[TpT]_0$ was kept constant at 10^{-2} *M*. $\Delta\delta_0$ is the difference between the chemical shift of the complexing TpT and the value of free TpT. The NMR spectra were measured in D_2O on a JEOL TNM-GX400 spectrometer at 30°C by use of *tert*-butanol as internal standard. Absence of measurable interaction of *tert*-butanol with the rare earth metal ions was confirmed by control experiments.

Preparation of Iminodiacetate-Attached DNA for Artificial Nuclease: A 19-mer DNA attached with an iminodiacetate at the 5'-end (DNA-IA) was prepared as depicted in FIG. 1. The 19-mer DNA having amino residue at the 5'-end was prepared on a CPG column by use of an automated synthesizer. The amino residue was activated by passing dry 1,4-dioxane solution of 1,1'-carbonyldiimidazole (30 *mM*, 10 ml) through the column. Then the column was treated with a dioxane solution of diethyl iminodiacetate (2 *M*, 10 ml) at 50°C. Between the reaction steps the reagents in excess and undesired products were washed out of the CPG column by dioxane. The ethyl esters were hydrolyzed by 10 ml of aqueous NaOH solution. In this step, the DNA derivative was detached from the CPG column. Finally the nucleic acid bases in the DNA derivative were deprotected by concentrated ammonia solution. After each of these steps a small portion of the product, removed from the column when necessary, was analyzed by anion-exchange HPLC (TOSOH DEAE-NPR column).

Sequence-Selective Scission of DNA by Ce(IV)-OligoDNA Hybrid: Mixture of the substrate 40-mer DNA and the DNA-IA was heated at 90°C for 2 min, and then was allowed to stand at 30°C for 1 h to complete the double helix formation between the two 19-mer sequences. Hydrolysis of the 40-mer DNA at pH 7.5 (50 *mM* Tris buffer) and 30°C was initiated by the addition of $Ce(NH_4)_2(NO_3)_6$ to the mixture. The reaction was followed by electrophoresis as described above.

RESULTS AND DISCUSSION

Hydrolysis of DNA Oligomer by Rare Earth Metal Ions: The electrophoresis patterns for the hydrolysis of the 40-mer DNA by various rare earth metal ions are depicted in FIG. 2. $LaCl_3$, $CeCl_3$, and $EuCl_3$ (other rare earth metal ions also) cleave the DNA. The catalysis by $CeCl_3$ is especially remarkable. The scission proceeds almost uniformly throughout the DNA chain: no specific base-preference is perceived. In con-

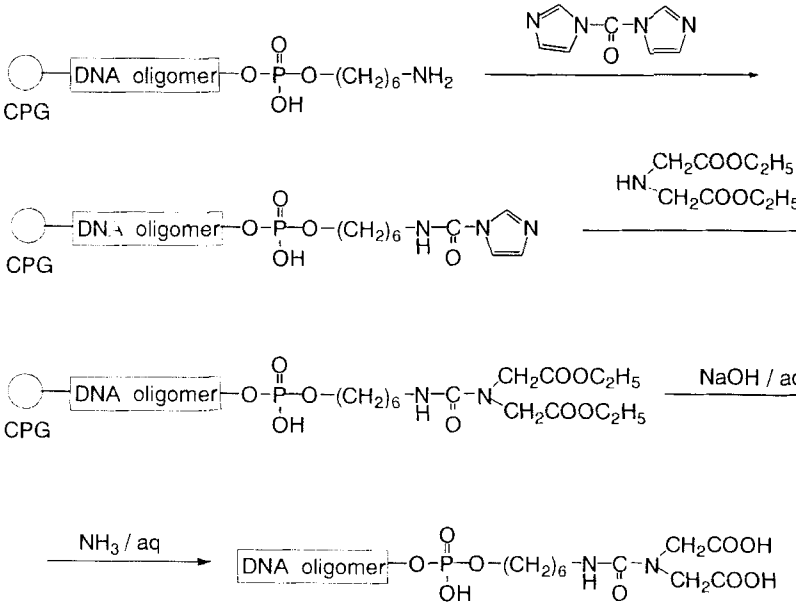

FIG. 1 Preparation of the DNA oligomer attached with an iminodiacetate residue (DNA–IA).

trast, non–rare earth metal ions Al^{3+}, Fe^{3+}, Mg^{2+}, and Zn^{2+} are totally inactive. Efficient catalysis is achieved only by rare earth metal ions.

Hydrolysis of Dinucleotide by Rare Earth Metal Ions: FIGURE 3 depicts the HPLC patterns for the hydrolysis of TpT by $CeCl_3$ at pH 7.0 and 50°C. TpT is promptly and stoichiometrically converted to two moles of thymidine (T). In the early stage, small amounts of pT and Tp are perceived, confirming the stepwise hydrolysis of phosphodiester linkage. Other rare earth metal(III) ions are also active for the conversion of TpT to T, although being much less efficient (1/100 or less) than $CeCl_3$. The pseudo first-order rate constant in the presence of $CeCl_3$ (0.01 *M*) is 2.0×10^{-1} (the half-life 3.5 h). $Ce(NH_4)_2(NO_3)_6$ also shows a large activity for the hydrolysis (the rate constant 1.7×10^{-1} h^{-1}). Neither thymine nor any other by-product is formed to a measurable extent. The possibility of concurrent oxidative cleavage of the ribose residue is ruled out, since thymine should be released if it were really the case. The present scission proceeds totally via the hydrolysis of the phosphodiester linkage.

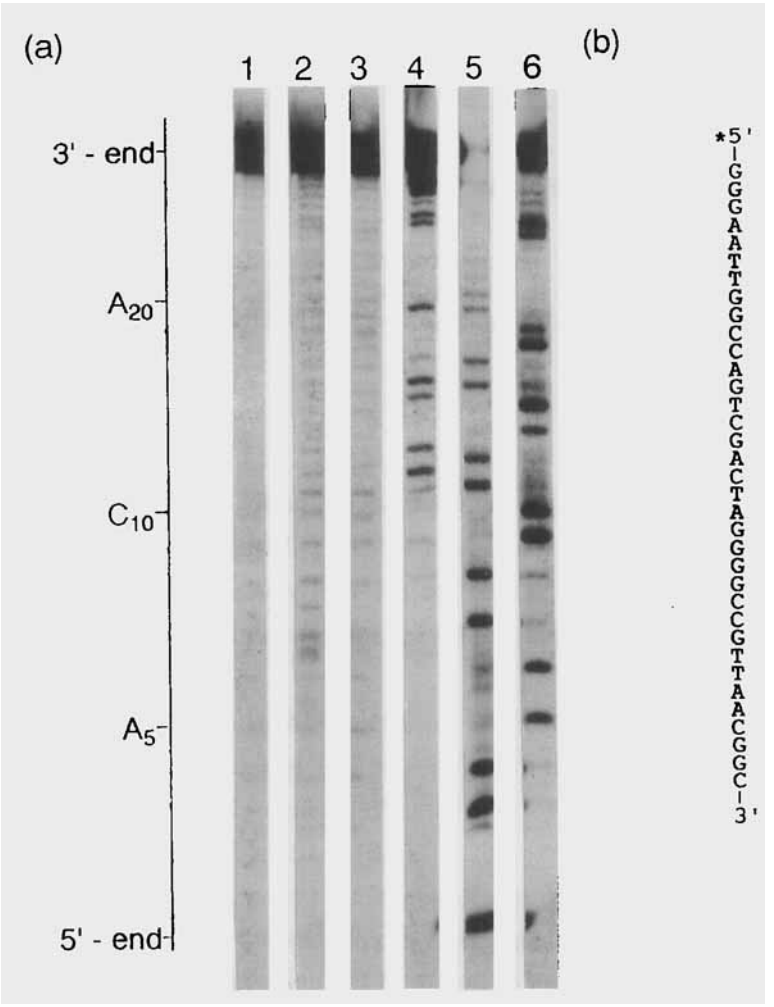

FIG. 2 Autoradiographs for the hydrolysis of the 35–mer DNA (shown in (b); ^{32}P–labeled at the 5'–end) by rare earth metal ions (10^{-2} *M*) at pH 7.2, 50°C : lane 1, $LaCl_3$; lane 2, $CeCl_3$; lane 3, $EuCl_3$; lane 4, digested by DNase I; lane 5, Maxam–Gilbert A+G sequencing reaction; lane 6, C+T sequencing reaction. The sequence scale shows the position of the fragment which has the designated deoxynucleotide at the 5'–end and has a 5'–phosphate terminus.

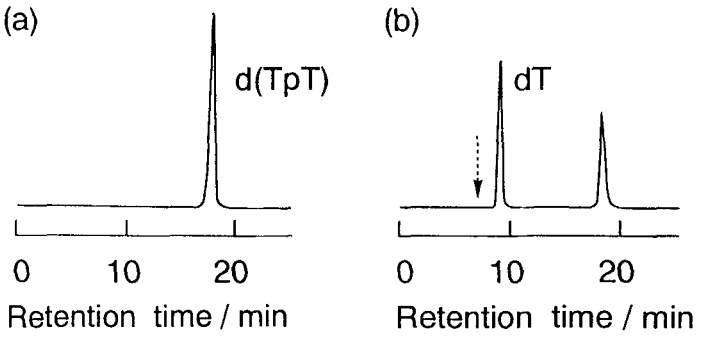

FIG. 3 Reversed-phase HPLC patterns for the hydrolysis of TpT by $CeCl_3$ at pH 7.0 and 50°C: (a) t = 0 h; (b) t = 3 h. The metal salt (10^{-5} *M*) was added to 1 ml of Hepes buffer, and the pH was adjusted by small amount of NaOH. The dotted arrow refers to the position of thymine.

Similarly d(ApA) was hydrolyzed to deoxyadenosine by $CeCl_3$ and $Ce(NH_4)_2(NO_3)_6$ (the rate constants are 4.7×10^{-2} and 4.8×10^{-2} h^{-1}). In the absence of rare earth metal ions, no hydrolyses take place at all. The pH–rate constant profile for the hydrolysis of TpT by $CeCl_3$ is a bell-shaped one having a maximum around pH 7.5 (FIG. 4).

Complex Formation between TpT and Rare Earth Metal Ion: Complex formation of TpT with Ce(III) ion shifted all the protons of TpT towards the lower magnetic field. The chemical shift changes ($\Delta\delta_{obs}$) are in the following order: 3' (4.7) > 5a" (4.4) > 5b" (4.2) > 2b' = 4' (2.2) > 2a' = 4" (1.1) > 1' (1.0) > others (the prime and the double-prime refer to the nucleoside in the 3'-side of the phosphodiester linkage and that in the 5'-side, respectively. The numbers in parentheses are the relative ratios of $\Delta\delta_{obs}$, which are virtually independent of the metal ion concentration. Only one complex species is formed in the mixture. Apparently the protons, which are located near the phosphodiester linkage, experience significant shifts due to the pseudo-contact shielding effects of the Ce(III) ion.[16] In contrast, the shifts for the 5-methyl protons in the thymine bases were marginal. A similar trend was observed for the complex formation between Eu(III) and TpT. Dominant complex formation of the rare earth metal ions with the phosphate residues in various ribonucleotide dimers rather than with the nucleic bases was shown previously.[16]

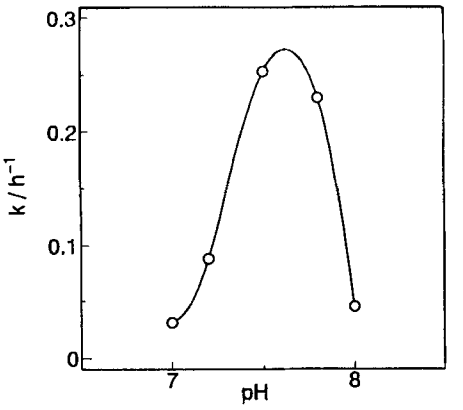

FIG. 4 pH–rate constant profile for the hydrolysis of TpT by $CeCl_3$ (10^{-2} *M*) at 50°C.

These results show that the Ce(III)–TpT complex involves coordination of the phosphate residue of TpT to the metal ion. The plots of $\Delta\delta_{obs}$ vs. $[Ce(III)]_0$ for the 3'–proton and the 5"a–proton satisfactorily fit the theoretical lines calculated according to eq. 1 using the same K value (30 M^{-1}: see FIG. 5 and TABLE 1). Note that eq. 1 is derived under the assumption that only 1:1 complex is formed.

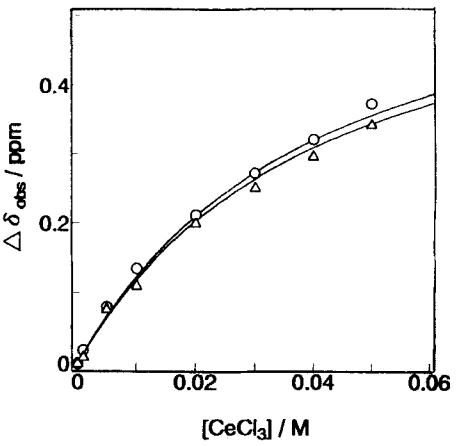

FIG. 5 Plot of $\Delta\delta_{obs}$ vs. $[CeCl_3]_0$ for the complex formation of TpT with $CeCl_3$: $[TpT]_0$ was kept constant at 0.01 *M*. (○), 3'H of TpT; (△), 5"a–H. The solid lines are the theoretical ones calculated by use of eq. 1 (K = 30 M^{-1}).

TABLE 1. Formation constants of complexes between TpT and rare earth metal ions at 30°C and pD 6.4.

Metal chloride	Complex formation constant / M^{-1}
$CeCl_3$	3×10^1
$EuCl_3$	8×10^1

Proposed Mechanism for the DNA Hydrolysis: The present hydrolysis proceeds via the complex between the phosphate residue of DNA and the rare earth metal ion. When $[TpT]_0 = 10^{-4}$ *M* and $[Ce(III)]_0 = 10^{-2}$ *M* at 30°C, about 20% of TpT is complexing with Ce(III) ion as estimated from the K value (30 M^{-1}) in TABLE 1. Assumedly the hydroxide ion bound to the metal ion intramolecularly attacks the phosphate coordinating to the same metal ion, as proposed for the hydrolyses of activated phosphate esters by lanthanide metal ions and complexes.[15,17] The water molecules coordinating to rare earth metal ions has a pK_a of 8–9.[18] Additionally the leaving group (the 3'– or the 5'–OH of deoxyribonucleoside) is stabilized by the water coordinating to the metal ion (or directly by the metal ion) as acid catalyst.[19] Furthermore, the positively charged metal ion electrostatically stabilizes the negatively charged transition state as the lysine–41 in ribonuclease A does.[20] Cooperation of these effects results in the remarkable catalysis of DNA hydrolysis, as schematically depicted in FIG. 6.

The decrease of the rate above pH 7.5 in the pH–rate constant profile (FIG. 4) is ascribed to the precipitation of the metal hydroxide at higher pH.

Ce(IV) Ion–OligoDNA Hybrid as Artificial Nuclease: A typical electrophoresis pattern for the scission of the 40–mer DNA (3'–labeled) by the Ce(IV) complex of the DNA–IA at pH 7.5, 30°C for 12 h is presented in FIG. 7 (lane 1). Densitometric analysis shows that the scission takes place mostly at the linkage between A31 and C32 (see the scission profile in (b)). The selectivity is 54% (the total conversion for the scission is 60 mol%). A highly selective and efficient artificial nuclease has been prepared. The scission is much more clear–cut than those for the artificial nucleases which cut DNA via the oxidative cleavage of the ribose.[2–4] This is ascribed to the fact that no diffusive species such as radicals are involving here.

The main product migrates more promptly than the Maxam–Gilbert fragment which ranges from A31 to A40 and has a 5'–phosphate terminus (at A31 in lane 2), but more slowly than the C32–A40 fragment of 5'–phosphate terminus (at C32 in lane 3). Note that the deoxyribonucleotide which is reacted in the base–specific Maxam–Gilbert reactions is ultimately removed by the subsequent treatments [21] (the sequence scale refers to the fragment which has the designated deoxynucleotide at the 5'–end). Thus the C32–A40 fragment having a 5'–OH terminus is dominantly formed by the present artificial nuclease (note that the DNA is labeled at the 3'–end).

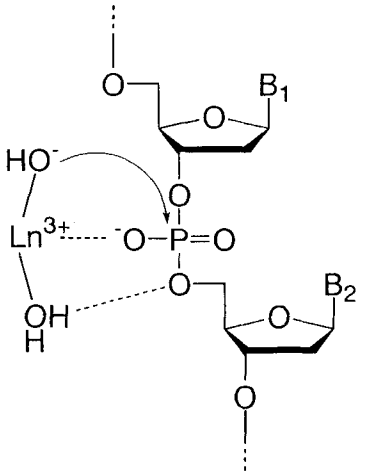

FIG. 6 Proposed mechanism for the DNA hydrolysis by rare earth metal ions.

When the 19-mer sequence in the DNA-IA and the complementary 19-mer sequence in the substrate DNA form a double helix, the Ce(IV) bound to the DNA-IA is placed near the linkage between A31 and C32. Thus the scission pattern is satisfactorily consistent with the molecular design. The minor cleavages at the other sites by the artificial nuclease are associated with the dangling motion of the single-stranded portion in the substrate DNA and/or fluctuation of the rather flexible hexamethylene linker in the DNA-IA (G20 is located almost beneath C32 in the double helix between the two 19-mer sequences and thus can be attacked by the Ce(IV)-iminodiacetate complex).

Use of Ce(III) and Eu(III) in place of Ce(IV) resulted in quite a poorer scission of the DNA. The large positive charge of Ce(IV) is required to achieve efficient catalysis. The metal ion in the iminodiacetate complex is surrounded by the negative charges of the ligand and thus its positive charge must be sufficiently large to activate the coordination water and also to stabilize the transition state for the hydrolysis electrostatically under these conditions.

CONCLUSION

Non-enzymatic hydrolysis of phosphodiester linkages in linear DNAs has been successfully achieved under physiological conditions by use of rare earth metal ions.

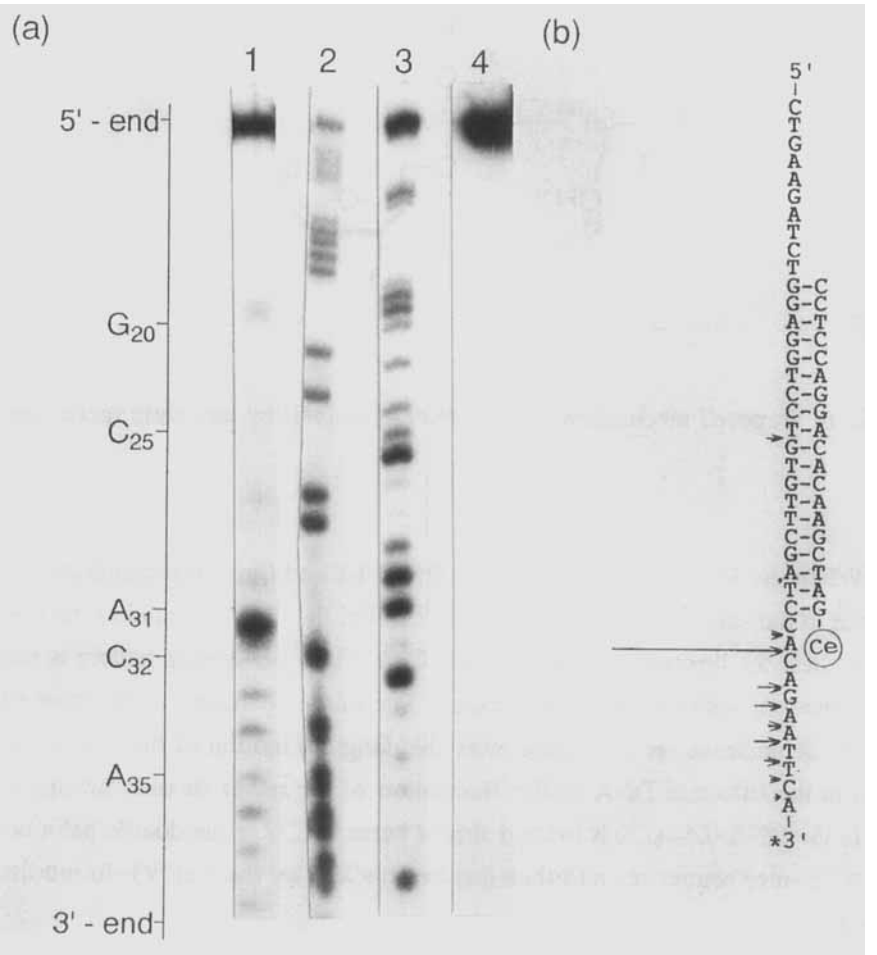

FIG. 7 Autoradiographs for the scission of the 40-mer DNA (shown in (b); ^{32}P-labeled at the 3'-end; $10^{-6}M$) by the combination of DNA-IA (10^{-5} *M*) and $Ce(NH_4)_2(NO_3)_6$ (10^{-5} *M*) at pH 7.2 and 30°C for 12 h (lane 1). lane 2, Maxam-Gilbert A+G sequencing reaction; lane 3, C+T sequencing reaction; lane 4, control. The sequence scale shows the position of the fragment which has the designated deoxynucleotide at the 5'-end and has a 5'-phosphate terminus. The scission profile is presented in (b). The Ce(IV)-DNA-IA hybrid was used in excess with respect to the substrate DNA so that the latter is mostly complexing with the Ce(IV)-DNA-IA hybrid.

The activities of Ce(III) and Ce(IV) ions are especially remarkable. No significant base-preference is perceived, indicating strong potentialities of these metal ions as the catalytic centers of artificial hydrolytic nucleases. The catalysis involves formation of the complex between the DNA and rare earth metal ion, in which the phosphate residue coordinates to the metal ion. Intramolecular attack by the hydroxide ion, which is bound to the metal ion, is indicated. An artificial nuclease has been prepared by the attachment of Ce(IV) to the 5'-end of the DNA oligomer by use of iminodiacetate ligand. The hybrid selectively cuts a DNA which has a 19-mer sequence complementary with the 19-mer portion in the hybrid. The scission by the artificial nuclease probably proceeds via the hydrolysis of the phosphodiester linkage as the scission by natural nucleases does.

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