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Circular Dichroism Studies of the HIV-1 Rev Protein and Its Specific RNA Binding Site†

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ABSTRACT: The circular dichroism (CD) spectrum of the Rev protein from HIV-1 indicates that Rev contains about 50% α helix and 25% β sheet at 5 °C in potassium phosphate buffer, pH 3, and 300 mM KF. The spectrum is independent of protein concentration over a 20-fold range. At neutral pH, Rev is relatively insoluble but can be brought into solution by binding to its specific RNA binding site, the Rev-responsive element (RRE), at a Rev:RNA ratio of about 3:1. Nonspecific binding to tRNA does not solubilize Rev. As judged by difference CD spectra, the conformation of Rev when bound to the RRE at neutral pH is similar to the conformation of unbound Rev at pH 3, although changes in the RNA may also contribute to the difference spectrum. Indeed, some difference is observed near 260 nm, consistent with a conformational change of the RRE upon Rev binding. Rev alone at pH 3 shows irreversible aggregation as the temperature is raised, while Rev bound to the RRE at neutral pH shows a reversible transition with a T_m of 68 °C.

Human immunodeficiency virus (HIV) encodes several regulatory proteins in addition to the Gag, Pol, and Env gene products encoded by most retroviruses. At least two of these regulatory proteins, Tat and Rev, are essential for viral replication (Fisher et al., 1986; Dayton et al., 1986; Sodroski et al., 1986; Terwilliger et al., 1988) and therefore are potential targets for anti-HIV drugs. Tat is a transactivator that increases expression of all viral genes and seems to function at both the transcriptional and posttranscriptional levels [see Cullen and Greene (1989) for a review]. Rev, a small protein of 13 000 daltons, appears to function posttranscriptionally (Malim et al., 1989; Hammarskjold et al., 1989) and is localized to the nucleus and nucleolus (Cullen et al., 1988; Perkins et al., 1989). In the absence of Rev, viral transcripts are fully spliced, resulting in production of both Rev and Tat proteins (Knight et al., 1987). Rev then increases the levels of incompletely spliced mRNAs that are transported to the cytoplasm, shifting viral protein synthesis toward production of the structural proteins (Sodroski et al., 1986; Feinberg et al., 1986; Knight et al., 1987; Sadaie et al., 1988; Malim et al., 1988; Hammarskjold et al., 1989). It has not yet been determined whether Rev acts by suppressing splicing (Feinberg et al., 1986; Emerman et al., 1989), by causing release of mRNAs from the spliceosome (Chang & Sharp, 1989), or by facilitating mRNA transport (Malim et al., 1989a; Felber et al., 1989; Hammarskjold et al., 1989).

The site of action for Rev is a highly conserved sequence located within the *env* gene, called the Rev-responsive element (RRE) (Malim et al., 1989a; Hadzopoulou-Cladaras et al., 1989; Rosen et al., 1988). This region forms a highly structured RNA element which must be maintained in the proper orientation to remain Rev-responsive (Dayton et al., 1989; Olsen et al., 1990; Malim et al., 1989a). Deletions within the RRE have defined at least one domain, the "hammerhead", as being necessary for Rev function (Dayton et al., 1989; Olson et al., 1990; Malim et al., 1990). Recently, several studies have shown that purified Rev binds with high affinity and

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specificity to RRE RNA in vitro (Daly et al., 1989; Zapp & Green, 1989; Cochrane et al., 1990b; Heaphy et al., 1990; Malim et al., 1990; Olsen et al., 1990).

To evaluate the specific nature of the Rev/RRE interaction and to facilitate the design of drugs specifically directed against Rev, the three-dimensional structure of Rev–RRE complexes will be needed. As an initial approach, circular dichroism (CD) has been used to examine the conformation of Rev alone and of Rev complexed to the RRE. In the present study, we show that Rev has a high α-helical content and that the conformation of Rev bound to the RRE is similar to unbound Rev. We also present data suggesting that the RRE may undergo a conformational change upon Rev binding.

Materials and Methods

Expression of Rev in Escherichia coli. The *rev* gene was cloned into the pREV 2.1 plasmid to generate the high-level expression plasmid pβGrev (Daly et al., unpublished results). pβGrev was transformed into *E. coli* RGN714 (Repligen Corp.) and grown in L-broth containing 20 μg/mL chloramphenicol at 40 °C in a 10-L Chemap fermenter. Cells were grown for 24 h with aeration at 10 L/min.

Purification of Recombinant Rev. Rev was purified under denaturing conditions as previously described (Daly et al., 1989), with modifications. Purification was monitored by Coomassie-stained SDS–PAGE and Western analysis using polyclonal antiserum developed against Rev purified from baculovirus-infected insect cells. Cells were lysed by physical disruption using a Dynomill cell disrupter. Rev present in the supernatant was ethanol-precipitated and resuspended in buffer containing 8 M urea. The sample was loaded onto a CM-Sepharose column equilibrated in low-salt buffer with 8 M urea and eluted with a 0–0.6 M NaCl gradient. Rev-containing fractions were pooled and chromatographed on a C_{18} reverse-phase HPLC column, and Rev was eluted with a 0–40% acetonitrile gradient in 0.075% trifluoroacetic acid. Rev was found to be greater than 95% pure by amino acid composition, N-terminal sequencing, and silver-stained SDS–PAGE. Some Rev samples were rechromatographed on an S-Sepharose column following HPLC and gave identical CD spectra. The specific activity of all protein preparations was determined by measuring dissociation constants of RRE binding using a nitrocellulose filter binding assay (Daly et al., 1989).

Synthesis of RRE-Containing RNA Fragments. As previously described (Daly et al., 1989), a cDNA clone containing the Rev-responsive element was inserted into the pBluescript vector (Stratagene) and transcribed in vitro using T7 RNA polymerase. Typically, 1 μg of linearized DNA template was incubated in 50 μL of 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, 200 μM each of ATP, CTP, GTP, and UTP, 15 mM DTT, 1 unit of Rnasin (Promega), and 10 units of T7 RNA polymerase. The transcription reaction was carried out at 37 °C for 30 min. The resulting 367-nucleotide-long fragment was purified with a Sephadex G-25 column, ethanol-precipitated, and resuspended at a concentration of approximately 1 μg/μL. The resulting RRE-containing RNA fragment was determined to be homogeneous by ethidium bromide staining following electrophoresis on a nondenaturing polyacrylamide gel. RRE concentrations were determined by absorption spectroscopy (A_{260} = 25.0 at 1 mg/mL).

Circular Dichroism. Circular dichroism spectra were measured with an Aviv Model 60DS spectropolarimeter, equipped with a Hewlett-Packard Peltier temperature controller. Spectra were recorded from 300 to 200 nm and averaged over five scans, with averaging times at each wavelength ranging from 10 to 60 s. Samples were prepared in 20 mM potassium phosphate buffer (adjusted to the appropriate pH with phosphoric acid or KOH) and 300 mM KF and centrifuged at 13000*g* for 5 min, and absorption spectra were recorded to determine Rev concentrations (A_{280} = 0.64 at 1 mg/mL). Rev concentrations varied between 16 and 320 μg/mL. All samples were degassed by vacuum prior to collection of the CD spectra, and spectra were recorded at 5 °C unless otherwise noted. Cylindrical cuvettes with path lengths of 1 or 5 cm were used depending on protein concentration, and dynode voltages were below 600 V at all wavelengths. Mean molar residue ellipticity was calculated using a molecular weight for Rev of 13051, and secondary structure was predicted from the spectra by using the PROSEC program (Aviv).

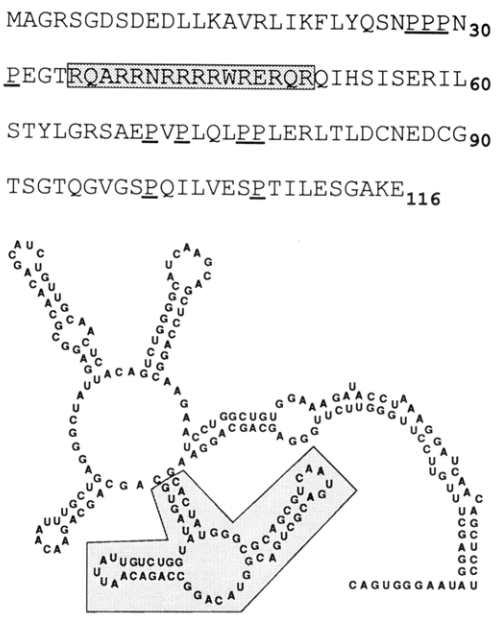

FIGURE 1: Sequence of the HIV-1 Rev protein and the Rev-responsive element (RRE). The Rev protein sequence is from the HIV-1 HXB-3 isolate [see Malim et al. (1989b)], and the arginine-rich region (boxed) and proline residues (underlined) are indicated. The RRE sequence is from the same HIV-1 isolate and is the secondary structure predicted by Malim et al. (1989a). The "hammerhead" region is shaded.

Results and Discussion

The sequence of the HIV-1 Rev protein (from isolate HXB-3) and the proposed secondary structure of the RRE RNA are shown in Figure 1. Rev contains an arginine-rich region (residues 35–50) and multiple proline residues scattered throughout its sequence. Mutational analyses suggest that the arginine-rich region is essential for Rev function and is important for nuclear localization (Cochrane et al., 1990a; Malim et al., 1989b), while the N-terminal 4 amino acids and C-terminal 16 amino acids are dispensable for activity (Malim et al., 1989b). Mutation of residues 78 and 79 (Leu-Glu) inactivates Rev and leads to a transdominant phenotype (Malim et al., 1989b). Genetic analyses (Dayton et al., 1989; Olson et al., 1990; Malim et al., 1990) and nuclease digestion patterns (Daly et al., unpublished results) suggest that the RRE adopts a highly ordered structure (Figure 1) and that the binding site for Rev is in the hammerhead region (shaded).

To examine the conformation of the Rev protein, we measured circular dichroism (CD) spectra at several pHs. Spectra obtained at pH 3 and pH 10.5 are shown in Figure 2. At pH 3, Rev shows considerable structure, with the double minima near 222 and 208 nm characteristic of α-helical conformation. The spectrum at pH 10.5 suggests that Rev is much less

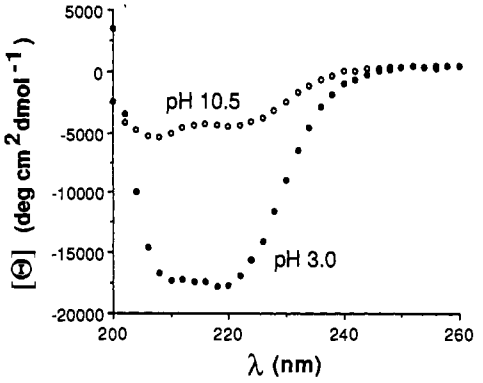

FIGURE 2: pH-dependent circular dichroism spectra of Rev. The concentration of Rev protein was 31 μg/mL, and spectra were recorded at 5 °C. Mean molar residue ellipticity was calculated by using a molecular weight of 13051 (116 amino acids).

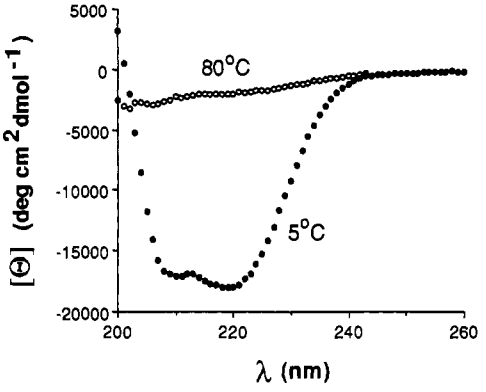

FIGURE 3: Circular dichroism spectra of Rev at pH 3. The concentration of Rev protein was 31 μg/mL at 5 °C. The 80 °C spectrum shown was not adjusted for the loss of protein due to precipitation and should not be considered the spectrum of unfolded Rev.

structured under these conditions. At intermediate pHs, particularly near neutral pH, the protein becomes insoluble. CD spectra of the pH 3 sample were also recorded at different temperatures (Figure 3). As the sample was heated above 50 °C, Rev showed a highly cooperative transition, but this unfolding led to irreversible aggregation (precipitation) and thus could not be used to calculate a T_m. The 80 °C spectrum shown in Figure 3 represents the fraction of protein remaining in solution and should not be considered the true spectrum of unfolded Rev. The temperature which caused Rev to precipitate was dependent on concentration, and precipitation was irreversible even at the lowest protein concentration tested (data not shown). The mean residue ellipticity of Rev at 222 nm (pH 3) showed no concentration dependence over a 20-fold range (16–320 μg/mL; data not shown), suggesting that the secondary structure was unaffected by any change in oligomeric state under these conditions. Protein secondary structure was estimated by fitting the pH 3 spectrum to basis spectra using the PROSEC program [a modification of the algorithm described by Chang et al. (1978)]. Rev was calculated to contain approximately 50% α helix, 25% β sheet, 25% random coil, and 0% β turn at pH 3. The high helical content seems to be near the upper limit possible since there are 10 proline residues spaced throughout the sequence (Figure 1). These secondary structure values are consistent with Chou and Fasman (1978) predictions.

We next examined the interaction of Rev with RNA containing the RRE. Previous studies by Daly et al. (1989) showed that Rev binds to the RRE with high affinity (K_d = 2×10^{-10} M) and specificity and that multiple Rev molecules bind to each RRE. We prepared complexes of Rev with the

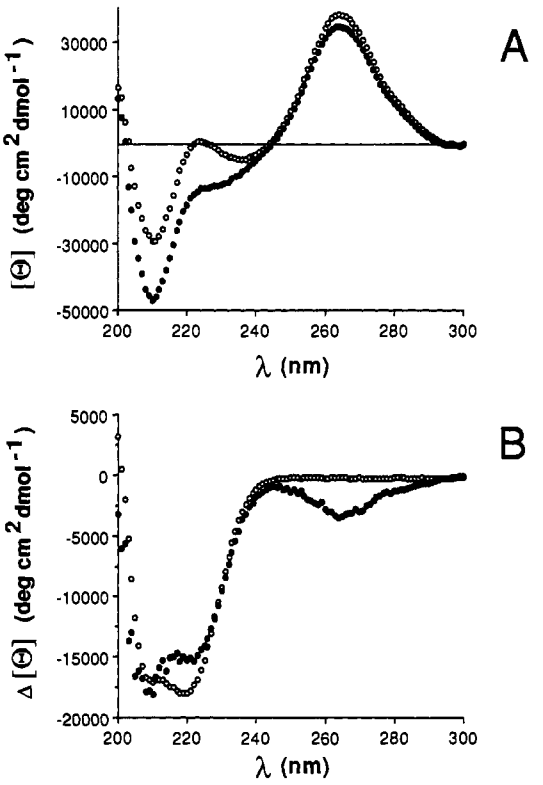

FIGURE 4: Spectra of RRE RNA and Rev–RRE complexes. (A) Spectrum of the RRE RNA alone at 50 μg/mL (○) or in the presence of 15 μg/mL Rev (●) at pH 7.4, 5 °C. The line at $[\theta] = 0$ shows that the RRE contributes no signal to the spectrum at 222 nm. (B) Difference spectrum of the spectra shown in (A) (●) overlayed with the pH 3 spectrum of Rev alone (○) (from Figure 3). To allow direct comparison of free and bound species, all spectra were normalized to the mean molar ellipticity of the peptide (see Figure 2). Coincidentally, the RRE spectrum in (A) also represents the mean molar ellipticity in nucleotides since the 3:1 molar ratio of Rev to RRE exactly compensates for the 1:3 amino acid to nucleotide molecular weight ratio.

RRE at 3:1 (Rev:RRE) stoichiometries at neutral pH and observed that, in contrast to Rev alone or Rev–tRNA complexes, Rev–RRE complexes were completely soluble. CD spectra of the RRE alone and of the Rev–RRE complexes are shown in Figure 4A. The spectrum of the RRE alone was virtually identical with the spectrum of tRNA (data not shown) and is similar to the spectra of other RNAs (Aboul-ela et al., 1988). To estimate the spectrum of the Rev component of the Rev–RRE complex, we subtracted the RRE spectrum from the complex spectrum (Figure 4B). This calculation assumes that the RNA does not undergo a significant conformational change that contributes to the difference spectrum below 250 nm. Indeed, double minima are seen near 210 and 222 nm and are similar to those seen for Rev alone at pH 3 (overlayed in Figure 4B). The calculated secondary structure from this difference spectrum (41% α helix, 17% β sheet, 42% random coil, and 0% β turn) suggests that the conformation of Rev bound to RNA is similar to unbound Rev at pH 3. To assess possible contributions from changes in RNA conformation, we recorded CD spectra of the RRE at 5 and at 80 °C and observed about a 50% decrease in the 260-nm maximum and a similar increase in the 240- and 210-nm minima (data not shown). No signal was seen at 222 nm under any condition (see Figure 4A). Thus, although there is some decrease in the calculated helical content of Rev upon RNA binding, it is difficult to assess how much of this difference is significant since changes below 220 nm may also result from changes in the RNA spectrum. The change in ellipticity at

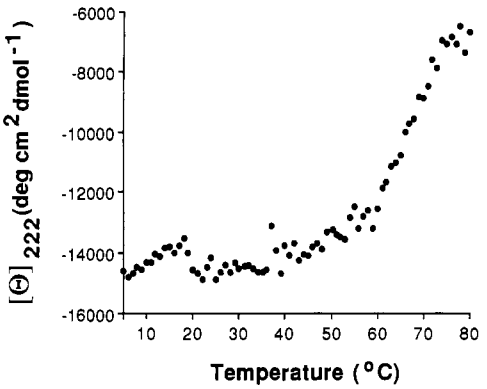

FIGURE 5: Melting curve of Rev bound to RRE. The Rev (10 μg/mL)–RRE (30 μg/mL) complex was heated from 5 to 80 °C with 60-s equilibration time per degrees centigrade and averaging for 60 s. The signal was monitored at 222 nm.

222 nm, however, can clearly be assigned to the protein. Interestingly, the difference spectrum also shows some change at 260 nm, corresponding solely to the signal from the RRE. This may represent a conformational change in the RRE as Rev binds and is consistent with localized melting of the RNA.

Since Rev and the RRE have very different spectra, it was possible to measure the melting curve for Rev in the Rev–RRE complex by monitoring the ellipticity at 222 nm where the RRE contributes virtually no signal (see Figure 4A). As previously described, in the absence of the RRE, Rev irreversibly aggregated upon heating at pH 3. In marked contrast, Rev bound to the RRE showed a sigmoidal melting curve (Figure 5) that was completely reversible and showed little hysteresis. The T_m of the transition was 68 °C, suggesting that the RNA-bound protein is very stable. The temperature dependence of the RRE signal in the absence of Rev, monitored at 260 nm, was also measured and showed a gradual decrease in intensity over a broad temperature range (data not shown). The broad, noncooperative transition suggests that the RRE contains regions of differing stabilities.

These CD studies indicate that the HIV-1 Rev protein contains significant α-helical and some β-sheet structure. By itself, the protein is highly soluble only at low pH (pH 3.0) or at high salt (>1 M NaCl), suggesting that protonation of carboxyl groups or shielding of charge may play an important role in protein solubility. This behavior of Rev is reminiscent of that seen with histones (D'Anna & Isenberg, 1973, 1974). Interestingly, specific binding to the RRE RNA completely solubilizes Rev at neutral pH, perhaps by affecting charged residues in a similar manner. Comparison of the CD spectra of Rev, the RRE, and the Rev–RRE complex suggests that the RNA may undergo a conformational change upon Rev binding. Such a change could be important for recognition of the RRE by cellular proteins necessary for Rev function. More detailed analyses of the interaction, including high-resolution RNA footprinting and NMR and crystallographic studies of the complex, will be necessary to fully understand the details of recognition of the RRE by Rev.

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Visible Light Induced DNA Cleavage by the Hybrid Antitumor Antibiotic Dynemicin A†

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ABSTRACT: Dynemicin A, which is a hybrid antitumor antibiotic containing anthraquinone and enediyne cores, effectively breaks DNA strands upon irradiation with visible light of long wavelength. The preferential cutting sites of visible light induced DNA cleavage with dynemicin A are on the 3′-side of purine bases such as in 5′-A*T* and 5′-G*T* sequences. The observed nucleotide cutting specificity is remarkably similar to that of NADPH- (or thiol) induced DNA breakage with dynemicin A, sugesting the presence of the same DNA-cleaving intermediate. Indeed, the photoproduct of dynemicin A is chromatographically identical with the reaction product (dynemicin H) of the thiol-activated dynemicin A. On the basis of the present results, a reasonable mechanism for the visible light induced DNA cleavage of dynemicin A has been proposed.

Dynemicin A isolated from *Micromonospora chersina* is characterized as a hybrid molecule of two typical chemotypes of antitumor agents, enediyne and anthraquinone. The novel antibiotic shows a potent antitumor activity in vitro and in vivo (Konishi et al., 1989, 1990). As to the mechanism of action of dynemicin A, we recently indicated that (1) DNA strand scission by dynemicin A is significantly enhanced by addition of NADPH or thiol compounds, (2) the antibiotic interacts with the minor groove of the DNA helix, and (3) intercalation of the anthraquinone core into the DNA followed by attack of the phenylene diradical formed from the enediyne core is most likely as the mechanism of action of dynemicin A (Sugiura et al., 1990). It is believed that DNA breakage by esperamicin, calicheamicin, and neocarzinostatin is due to similar phenyl diradical species produced from their enediyne cores (Long et al., 1989; Sugiura et al., 1989; Zein et al., 1988; Myers, 1987; Goldberg, 1987). Thiol compounds such as dithiothreitol and glutathione greatly accelerate the cleavage of DNA by esperamicin, calicheamicin, and neocarzinostatin. Ultraviolet light induced DNA nicking has been observed for the cobalt, manganese, and iron complex systems of bleomycin (Suzuki et al., 1985). In the case of esperamicin or neocarzinostatin, irradiation with ultraviolet light is also effective in causing nicks in supercoiled DNA (Uesawa et al., 1989). In this paper we report that visible light induces remarkable DNA cleavage by dynemicin A and that the photoproduct of dynemicin A is chromatographically identical with the reaction product (dynemicin H) of dynemicin A and thiol compounds.

Materials and Methods

Drugs and Chemicals. Purified dynemicins A, L, and H (Figure 1) were kindly supplied by Bristol-Myers Research Institute, Tokyo, Japan. Plasmid pBR322 DNA was isolated from *Escherichia coli* C600, and restriction endonucleases *Eco*RI and *Hae*III were obtained from Takara Shuzo (Kyoto, Japan). G4 DNA obtained from phage R199/G4 ori replicative form DNA was a kind gift of T. Komano (Kyoto University, Kyoto, Japan). NADPH and 4-hydroxythiophenol were purchased from Sigma. All other chemicals used were of commercial reagent grade.

Assay for DNA Cleavage Activity. Analysis of drug-induced damage to supercoiled, covalently closed, circular (form I) pBR322 DNA was performed under irradiation of visible light. The reaction samples (total volume 20 μL) contained 20 μM dynemicin A, 0.4 μg of pBR322 plasmid DNA, and 20 mM Tris-HCl buffer (pH 7.5) and were irradiated at a distance of 10 cm by a commercial 300-W photoreflector lamp (Toshiba, Tokyo; color temperature of 3150 K) for 30 min at 4 °C. To investigate the dependence on wavelength of visible light for DNA cleavage activity, various photofilters were used. UV, Y2, R1, and R64 filters (Kenko, Tokyo) approximately cut off wavelengths shorter than 360, 460, 580, and 610 nm, respectively. The reactions were stopped by addition of cold ethanol (60 μL) and 0.3 M sodium acetate, then the samples were immediately chilled at −70 °C in a dry ice/ethanol bath, and the DNA was recovered by ethanol precipitation. Each of the samples was dissolved in 20 μL of loading buffer containing 0.05% bromophenol blue and 10% glycerol and heated for 1 min at 60 °C before electrophoresis. Electrophoresis was peformed on a 1% agarose gel containing ethidium bromide (0.5 μg/mL).

Nucleotide Sequence Analysis. The reaction samples (total volume 20 μL) contained a 5′ or 3′-end-labeled 100-base-pair (bp) (*Eco*RI–*Hae*III) G4 gene F/G space fragment, sonicated calf thymus carrier DNA (5 μg/mL), and 20 mM Tris-HCl buffer (pH 7.5). Nucleotide sequence cleavage was performed by addition of dynemicin A (20 μM) and NADPH (1 mM) or 4-hydroxythiophenol (1 mM) for 6 h at 37 °C or by irradiation of visible light through the UV filter for 30 min at 4 °C. The condition of light exposure was the same as described

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