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EVIDENCE FOR A SITE-SPECIFIC ENDONUCLEASE IN YEAST MITOCHONDRIA WHICH RECOGNIZES THE SEQUENCE 5'GCCGGC

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We have discovered a mitochondrial, site-specific DNase in *Saccharomyces cerevisiae* with properties like that of a Type II restriction endonuclease. The enzyme, termed ScelII, cleaves the palindromic sequence, 5'GCCGGC, to give 3' ends recessed by 4 bases. ScelII is the first restriction-like endonuclease to be described in yeast mitochondria.

Several site-specific endonucleases have been described in yeast mitochondria (1,2,10). Some of these are encoded by group I introns and recognize and cleave a single transpositional target site (1,10) The site-specific endonuclease, Endo-SceI, is present in mitochondria of some yeast strains and consists of a heterodimeric protein (2). The larger subunit is encoded by a nuclear gene (ENS1) while the smaller subunit is encoded by a mitochondrial open reading frame (ENS2) that is optional among yeast strains. The intron-encoded endonucleases and endo-SceI are different from prokaryotic restriction endonucleases in that they recognize and cleave DNA at sites containing an extended recognition sequence with no dyad symmetry. We have discovered a site-specific endonuclease activity in yeast mitochondria with properties like those of a Type II restriction endonuclease. The endonuclease, termed ScelII, recognizes the palindromic

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sequence 5'GCCGGC and produces 3' ends recessed by 4 bases. This is the first time a type II-like restriction endonuclease has been described in yeast mitochondria.

MATERIALS AND METHODS

Site-Specific Endonuclease Assay

To detect nuclease activities in mitochondrial extracts, a dimeric form of plasmid DNA (pH101) was used as substrate. This plasmid contained a 650 bp HpaII DNA fragment from the upstream and the amino terminal portion of the yeast mitochondrial VAR1 gene cloned into the AccI site of pBS(-) plasmid (Stratagene, La Jolla, CA).

Mitochondrial extracts were prepared (3) from strain A1237m161 (*a ade1 ura3 leu2 nuc1-1*) which lacked the major mitochondrial nuclease. Partial purification of mitochondrial DNase activities was done as described previously (4). During the purification procedure, two different DNases were detected: a nonspecific nicking activity, termed mtDNase I, and the site specific endonuclease activity, SceIII. Both of these activities co-eluted through the final fractionation step of DNA-cellulose chromatography. However, preincubation of the enzymatic fractions containing mtDNase I and SceIII at 55°C for 2 hrs inactivated the majority of mtDNase I activity but did not significantly decrease SceIII activity.

Therefore, the standard protocol for measuring SceIII activity was to preincubate a reaction mixture (20 µl) containing 25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and column fractions containing SceIII at 55°C for 2 hrs, after which the reaction was initiated by adding 1.25 µg supercoiled plasmid DNA as the substrate. The reaction mixture was incubated at 37°C for 5 hrs and then enzymatic activity was terminated by addition of 10 µl of 4% SDS, 40 mM EDTA, 40% glycerol and 0.02% bromophenol blue. The samples were electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide and the DNA bands were visualized by UV light (302 nm).

Analysis of the DNA Sequence of the SceIII Cleavage Site

Plasmid pH101 DNA was digested with NdeI (which has a single site in pH101) and the DNA ends were 5' end-labeled with [α-^{32}P]ATP and T4 polynucleotide kinase (5). The labeled DNA was digested with EcoRI, which gave singly end-labeled DNA fragments of 0.7 kb and 3.1 kb. The 0.7 kb fragment, which contained the cleavage site for SceIII, was gel purified and digested with SceIII. Another aliquot of the 0.7 kb DNA was subjected to base-specific chemical cleavages (6). Both of these reacted DNAs were subjected to electrophoresis in a sequencing gel under denaturing conditions to map the cleavage site of SceIII by comparison of the length of the reacted product to the sequencing ladder.

Densitometry

Photographic negatives of an ethidium bromide-stained gel were scanned using a Molecular Dynamics Personal Densitometer which converts

the data into a digital representation. Area integration along a line positioned down the center of the individual gel lanes was performed to generate a tracing of density versus distance from a common origin (positioned slightly below the gel well). The tracings were manually superimposed for a better comparison of the reaction products resulting from ScelII versus NaeI digestion of the DNAs.

RESULTS

To measure ScelII activity, we used plasmid pH101 in the form of a circular dimer containing head to tail repeating units. Incubation of pH101 with mitochondrial extracts yielded linear monomers and dimers consistent with an incomplete digestion at a specific site (Fig. 1). To map that site we digested ScelII reaction products with HindIII and EcoRI. The pattern of digestion products revealed that ScelII cleaved in the vector portion of the pH101 ~500 bp from the EcoRI site.

ScelII was active in a narrow pH range (7.5-9.0) with maximal activity at pH 8.0. The enzyme required either Mg^{++} or Mn^{++} for activity which was maximal at 10 mM Mg^{++} or 5 mM Mn^{++}. Activity was not supported by Ca^{++} and Zn^{++}. ScelII activity was inhibited by concentrations of NaCl or KCl above 20 mM and was maximal in reactions incubated between 40°-50°C. ScelII co-localized with gradient-purified mitochondria that had been treated with digitonin to remove their outer membranes (3).

To determine the sequence of the ScelII cleavage site, we used pH101 DNA that had been 5' end-labeled at the NdeI site (Fig. 1). Fig. 2 shows that the 3' end of the labeled ScelII cleavage product mapped within the palindromic sequence, 5' GCCGGC. The ScelII product band co-migrated with the band derived from chemical modification of and cleavage at the second C residue of that sequence. Since chemical sequencing reactions eliminate the modified base upon cleavage (6), the enzymatic cleavage apparently occurred between the G and C residues as indicated in the figure. Consistent with this conclusion, ScelII cleavage products were labeled by Klenow DNA PolI in a fill-in reaction containing unlabeled dCTP plus [α-^{32}P]dGTP but not one containing only labeled dGTP (data not shown). With a 3' end-labeled NdeI fragment, cleavage of the complementary strand occurred between the last G and C residues (data not shown). Thus, ScelII cleaved DNA to yield 3' ends that were recessed by 4 bases and terminated with a hydroxyl group.

The apparent recognition sequence of ScelII is also recognized by the restriction endonuclease NaeI. To determine whether that recognition sequence was necessary and sufficient for cleavage, we reacted various DNAs with ScelII and compared their digestion patterns to those generated by NaeI. There are no NaeI sites in øX174, one in phage λ, and four in pBR322.

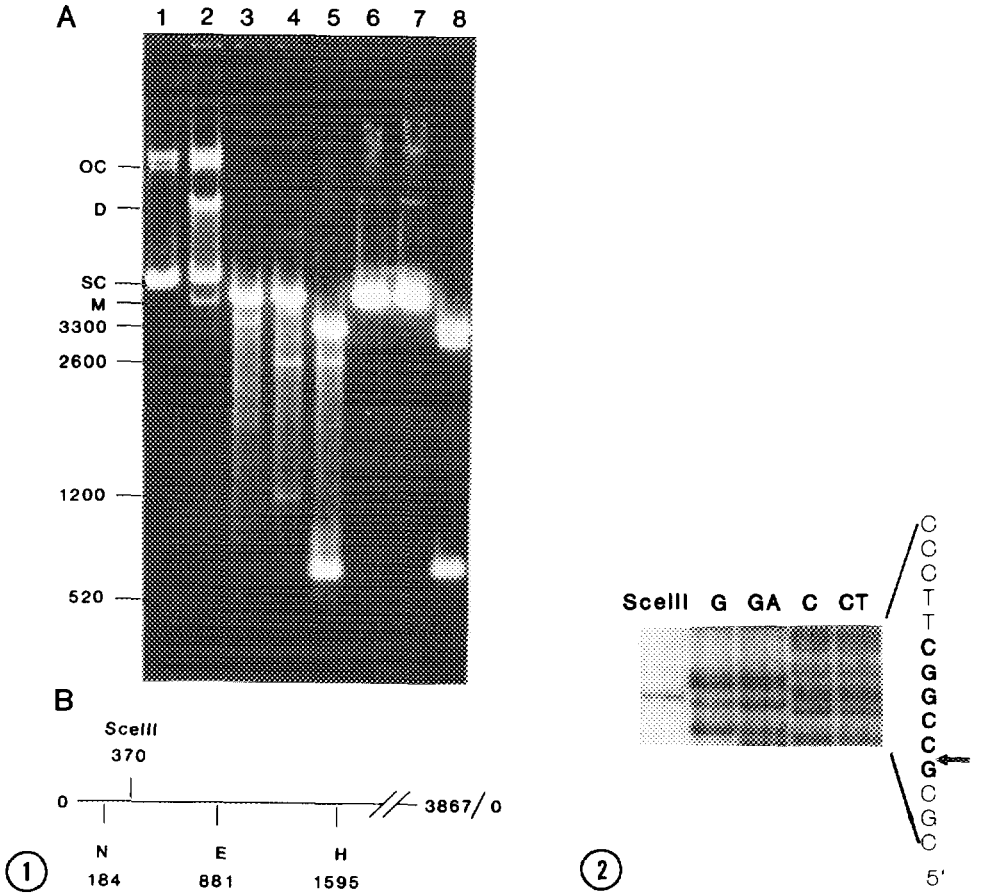

FIGURE 1. Mitochondria contain a site-specific endonuclease. Panel A: A partially purified mitochondrial extract (containing ScelII activity) was incubated with 1.25 µg pH101 DNA for 2.5 hrs at 37°C (lane 2). Following incubation, the DNA was phenol-extracted, ethanol-precipitated, and further digested with EcoRI (lane 3), HindIII (lane 4), and EcoRI + HindIII (lane 5). Lanes 6, 7 and 8 show control digestions with EcoRI, HindIII, and EcoRI + HindIII, respectively; lane 1: undigested pH101. Indicated on the left are the positions of linear monomeric (M) and dimeric (D), supercoiled (SC), and open circular (OC) pH101 DNAs. From the lengths of the ScelII-specific restriction fragments (indicated on the left), the ScelII cleavage site was located on the pH101 map (Panel B; E: EcoRI, H: HindIII, N: NdeI).

FIGURE 2. Sequence of the ScelII cleavage site. An NdeI + EcoRI restriction fragment of pH101 (Fig. 1), 5' end-labeled at the NdeI site, was reacted with ScelII. The product was electrophoresed through a denaturing gel next to a chemical sequencing ladder (base-specific reactions indicated) of the same fragment. The cleavage site for ScelII between the indicated G and C residues is shown by the arrow.

With PstI-linearized øX174 DNA, no NaeI cleavage products were detected, as expected, and similarly, none were seen upon ScelII digestion (Fig. 3). It was evident that the ScelII preparation contained some non-specific endonuclease

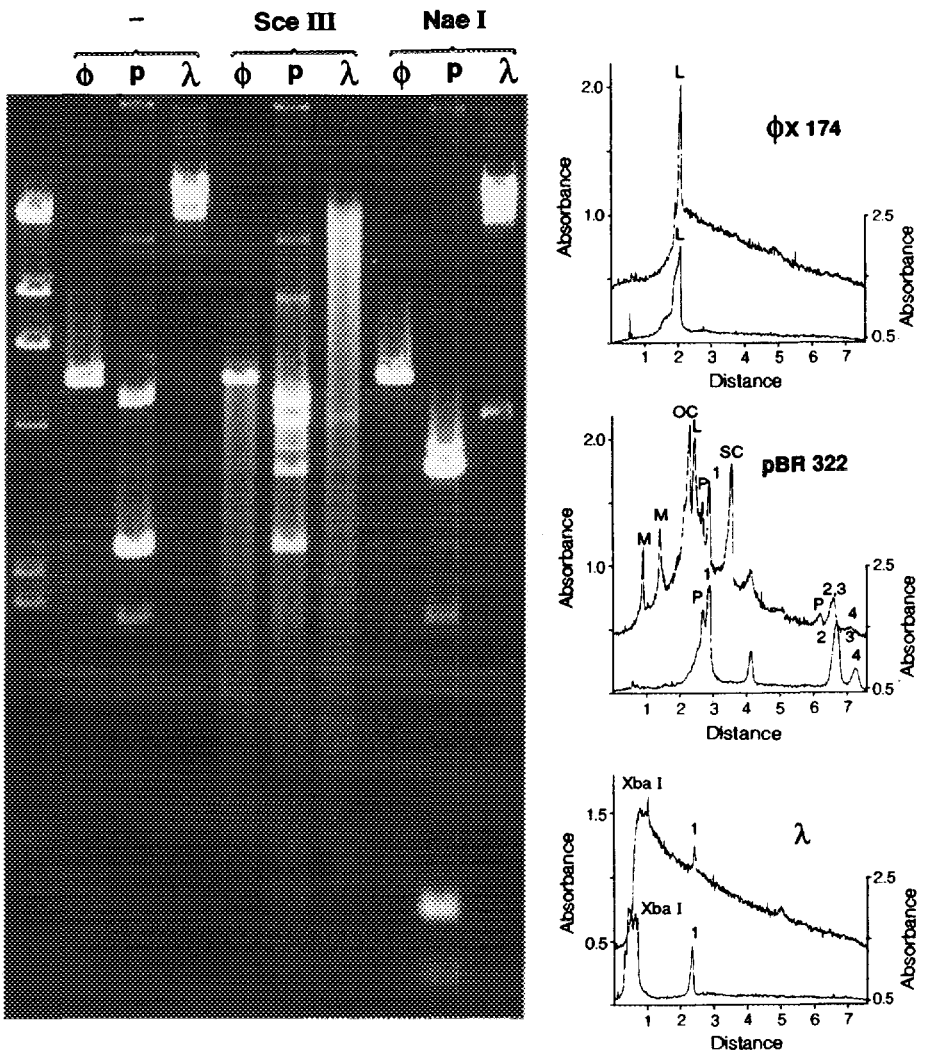

FIGURE 3. SceIII and NaeI have identical sequence specificity for DNA cleavage. DNAs from øX174 (ø; linearized by PstI digestion), pBR322 (p), and phage λ (λ; digested with XbaI) were reacted with no enzyme (-), SceIII, and NaeI, as indicated, fractionated by agarose gel electrophoresis, and then visualized by ethidium bromide staining. The first lane on the left shows a HindIII digestion of phage λ DNA. The lanes containing enzymatic reaction products were analyzed by densitometry and the superimposed scan tracings are shown on the right. In each panel the top tracing is from the lane containing SceIII reaction products. Note that the absorbance scale (in arbitrary units) used for the SceIII tracing (left axis) is different from that for the NaeI tracing (right axis). Abbreviations: L, linear; OC, open circular; SC, supercoiled; M, multimer; P, partial digestion product. The numbers labeling peaks in the tracings indicate predicted digestion products. The faint band in the lanes containing pBR322 DNA migrating at distance marking 4 was a contaminant.

activity, as indicative in Fig. 3 by the smear of fluorescent material migrating faster than the linearized øX174 DNA band. In order to detect with greater sensitivity digestion products which may be obscured by the smear, the gel photographic negative was analyzed by densitometry. To the right in Fig. 3 are presented densitometric tracings of the gel lanes containing either NaeI or SceIII reaction products (in each panel the top tracing shows the results using SceIII). The densitometric analysis of SceIII-reacted øX174 DNA showed that no specific digestion products were observed, indicating that no SceIII digestion sites were present in øX174 DNA.

Phage λ DNA has one NaeI site approximately 4500 base pairs distant from the single XbaI site located in the middle of the linear phage genome. Cleavage of XbaI digested λ DNA with either NaeI or SceIII each yielded the same 4.5 kbp product band (Fig. 3). The densitometric tracing of the fractionated reaction products showed no other product bands indicating that NaeI and SceIII each cleaved phage λ DNA at the same position within the resolution of the gel analysis. Note that the small peak observed in the tracing of the SceIII reaction products at distance marking 5 was also present in the tracings of the SceIII reactions with øX174 and pBR322 DNAs; thus, it did not represent a substrate-specific digestion product.

Using pBR322 DNA, the entire spectrum of NaeI digestion products was also observed with SceIII, including the prominent partial NaeI reaction product labeled "P" in the densitometric tracing of the NaeI gel lane (Fig. 3). This partial was a fusion of bands 1 and 3 based upon the known slow reactivity of NaeI with its site at position 1283 in pBR322. In the SceIII reaction we also observed a smaller partial reaction product (peak labeled "P" in the SceIII tracing at distance marking 6) which was the expected size of a fusion of bands 4 with either 2 or 3. The other bands observed in the SceIII reaction of pBR322 DNA were also present in the control lane (Fig. 3, minus enzyme); their presence reflected the low enzymatic activity of the SceIII preparation. Together, these three DNAs contained all single base variations of the sequence, 5'GCCGGC and all pair-wise variations that retain palindromic character of the four unchanged bases. From the data presented here we conclude that the sequence 5'GCCGGC is both necessary and sufficient to serve as a cleavage site for SceIII.

DISCUSSION

In the present study we have identified and characterized a site-specific endonuclease (SceIII) that is found in the mitochondria of yeast. Although other site-specific endonucleases have been identified in yeast (7,8,9), some of which are located within the mitochondria (2,10), SceIII is

the first to show properties of a type II restriction enzyme. In the green alga, *Chlorella*, restriction endonucleases have also been found but in this case they have been encoded in the genome of a virus which infects *Chlorella* (11). SceIII recognizes and cleaves at the palindromic sequence, 5'GCCGGC, so as to give a four base recessed 3' end terminated with a hydroxyl group. Although this sequence is necessary and sufficient for SceIII cleavage, the possibility remains that the flanking DNA sequences could significantly affect the reactivity of the enzyme. The role of this enzymatic activity in mitochondria is unknown.

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REFERENCES

1. Dujon, B., and Belcour, L. (1984) In Mobile DNA (D. E. Berg and M. M. Howe, Eds.), pp. 861-878. American Society of Microbiology, Washington D.C.
2. Nakagawa, K., Morishima, N., and Shibata, T. (1991) J. Biol. Chem. 266, 1977-1984.
3. Dake, E., Hofmann, T. J., McIntire, S., Hudson, A., and Zassenhaus, H. P. (1988) J. Biol. Chem. 263, 7691-7702.
4. Min, J. J., Heuertz, R. M., and Zassenhaus, H. P. (1993) J. Biol. Chem. 268, 7350-7357.
5. Maniatis, T., Fritsch, E. F., and Sabbrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor University Press, Cold Spring Harbor, NY.
6. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
7. Watabe, H., Shibata, T., and Ando, T. (1981) J. Biochemistry 90, 1623-1632.
8. Kostriken, R., Strathern, J. N., Klar, A. J., Hicks, J. B., and Heffron, F. (1983) Cell 35, 167-174.
9. Kostriken, R., and Heffron, F. (1984) Cold Spring Harbor Symp. Quan. Biol. 49, 89-96.
10. Perlman, P. S., and Butow, R. A. (1989) Science 249, 1106-1109.
11. Van Etten, J. L., Lane, L. C., and Meints, R. H. (1991) Microbiol. Rev. 55, 586-620.