Free Radical Research, 2002 VOL. 36 (5), pp. 491–498

Taylor & Francis
healthsciences

Identification of a Single Genome by Electron Paramagnetic Resonance (EPR) with Nitroxide-labeled Oligonucleotide Probes

JEFF D. HESTER[a,b], ELIZABETH V. BOBST[a], DAVID D. KRYAK[b] and ALBERT M. BOBST[a,*]

[a]*Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221-0172, USA;* [b]*National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268-1320, USA*

Accepted by Professor H. Sies

(Received 2 October 2001)

Nitroxide-labeled nucleic acids are used as a molecular size sensor to identify as few as one genome under polymerase chain reaction (PCR) conditions by electron paramagnetic resonance (EPR) spectroscopy. DNA identification is based on differences in the EPR spectra of mono-nitroxide-labeled nucleic acids. The experimental data imply that rapid DNA identification can be achieved in many systems by EPR at the molecular level.

Keywords: Nitroxide dynamics; Oligonucleotides; Electron paramagnetic resonance; Genome

Abbreviations: Probe, nitroxide-labeled oligonucleotide; "Flexi" nitroxi, nitroxides weakly coupled to biopolymer by covalent linkage; "Rigi" nitroxides, nitroxides strongly coupled to biopolymer by covalent linkage; SDSL, site-directed spin labeling; T_m, annealing temperature

INTRODUCTION

Spin-labeled nucleic acids have been used to study structural and dynamic features of DNA by electron paramagnetic resonance (EPR) spectroscopy (for a recent review see).[1] A systematic analysis of EPR spectra of DNA spin-labeled with differently tethered nitroxides was achieved recently with the slowly relaxing local structure (SRLS) model,[2] wherein the nitroxide is reorienting in a restricted local environment, which itself is relaxing on a longer time scale. Our laboratory has mainly worked with long-tethered ("flexi") nitroxides to avoid perturbation of the DNA structure common with short-tethered ("rigi") nitroxides. As early as 1978, "flexi" nitroxides were being enzymatically incorporated into nucleic acids,[3] while subsequent studies revealed that "rigi" nitroxides were poor substrates for polymerases.[4] The incorporation of "flexi" or "rigi" nitroxides into nucleic acids with enzymes or chemical synthesis produced spin-labeled nucleic acids with EPR line shapes that have been the subject of numerous analyses and discussions.[2,5]

In the present study, we show the feasibility of using changes in the nitroxide dynamics of spin-labeled nucleic acids in the fast motional range to identify one or more DNA molecules by EPR. Instead of relying on the polymerase activity to incorporate "flexi" nitroxides into nucleic acids, the $5' \rightarrow 3'$ nuclease activity of *Thermus aquaticus (Taq)* DNA polymerase[6] is used to form nitroxide-labeled fragments from the nitroxide-labeled oligonucleotides (probes). The enzymatic release of these fragments from the probes generates a population of faster tumbling species with smaller correlation times readily detectable by EPR. Under polymerase chain reaction (PCR) amplification conditions, enough nitroxide-labeled fragments are generated so that no more than one genome is required for its identification.

*Corresponding author. Tel.: +1-513-556-9281. Fax: +1-513-556-9239. E-mail: bobst@uc.edu
This work was presented at the 3rd International Conference on Nitroxide Radicals, September 23–29, 2001 in Kaiserslautern, Germany.

ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10715760290025870

The feasibility of the concept was explored with the genome of *Encephalitozoon hellem*, which is one of 14 microsporidial species from 7 genera currently reported to be etiological agents of disease in humans. Probes with different nitroxide groups and attachment sites obtained by site-directed spin labeling (SDSL) were used to identify one or more *E. hellem* spores and to discriminate them from spores of *E. cuniculi* and *E. intestinalis*. The preliminary results reported here suggest that nitroxide-labeled nucleic acids can serve as a molecular size sensor for the identification of DNA from various sources at the molecular level.

MATERIAL AND METHODS

Microsporidial Spores

E. hellem (CDC: 0291:V213), *E. cuniculi* (ATCC#50502) and *E. intestinalis* (ATCC#50603) spores were cultured, harvested and purified as previously reported.[7]

Design of Spin-labeled Probes and Primers

Microsporidial 16S rRNA gene sequences were obtained from the NCBI GenBank database and aligned with the MEGALIGN software package (DNASTAR, Incorporation, Madison, WI). Genetically conserved regions were identified for the genus *Encephalitozoon* that were flanked by genetically variable regions with respect to the phylum Microsporidia. The selection of an *Encephalitozoon*-specific spin-labeled probe and an *E. hellem*-specific primer set was made with the ABI Primer Express® program (Applied Biosystems, Foster City, CA). In order to verify specificity, the nucleic acid sequence of the probe and primer set were checked against all available nucleic acid sequences in the NCBI GenBank database.

Oligonucleotide Synthesis of Primers and Spin-labeled Probes

Oligonucleotide primers and probes were synthesized on a Perkin Elmer-Applied Biosystems 381A DNA synthesizer using standard phosphoramidite chemistry. All oligonucleotide probes were chemically phosphorylated in position 3′ with a 2-[2-(4,4′-dimethoxytrityloxy) ethylsulfonyl]ethyl-2-succinoyl)-long chain alkylamino CPG column (Glen Research, Sterling, VA). The unlabeled precursor of ENSP was obtained with 6-(trifluoroacetylamino)-hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (5′-Amino-Modifier C6-TFA) (Glen Research) and then spin-labeled with 2,5-dioxypyrrolidin-1-yl 2,5-dihydro-2,2,5,5-tetramethyl-1-oxyl-1*H*-pyrrole-3-carboxylate using a published procedure.[8] The unlabeled precursor of ENBP was prepared with 5′-dimethoxytrityl-5- [*N*-(trifluoroacetylaminoethyl)-3-acrylimido]-2′-deoxyuridine,3′-[2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (Amino-Modifier C2 dT) (Glen Research) and then spin-labeled by the same method used for ENSP. EDUP was synthesized in one step with the phosphoramidite derivative of DUMTA prepared with a published protocol used for the synthesis of the DUTA phosphoramidite.[9]

Oligonucleotide Purification

Depending on the properties of the modified oligonucleotides different protocols and columns were required for optimal purification with an FPLC instrument and columns from Amersham Pharmacia (Uppsala, Sweden) resulting in capillary electrophoresis (CE) pure products.[1] The unlabeled precursor of ENSP, the unlabeled tritylated ENBP and tritylated EDUP were purified on a 3-ml Resource RPC column, and after spin-labeling, ENSP and ENBP were put through a Mono Q 5/5 anion exchange column. After a final desalting step on a Fast Desalting HR 10/10 column the A_{EPR}[1] of all probes was determined before storing them at −20°C. The unlabeled tritylated EhelF1 and EhelR2 primers were purified on a 3-ml Resource RPC column and then stored at −20°C.

Enumeration of Microsporidial Spores and Isolation of DNA

Flow cytometric counting of microsporidial spores was performed with a FACSVantage SE (Becton Dickinson, San Jose, CA) equipped with an argon laser (488 nm) and Clone-Cyt software (Becton Dickinson). FACSFlow isoton (Becton Dickinson) was used as sheath fluid. The spores were gated and sorted by their forward (FSC) and side (SSC) scatter profile (FSC gain 2, SSC gain 1 352 V, and FSC threshold 26 V). Spores were sorted into a 1.5 ml centrifuge tube containing 0.1 ml reagent water. A predetermined number of spores from each of the three *Encephalitozoon* species were flow counted and known amounts of spores per 100 μl were used for the DNA extraction procedure. A suspension of counted microsporidial spores were added to a capped 2 ml conical bottom screw-cap tube containing 0.35 g of autoclaved, acid-washed glass beads (425–600 μm, Sigma–Aldrich Company, St. Louis, MO) and 180 μl of lysis buffer of the DNeasy™ Tissue Kit (Qiagen, Incorporation, Valencia, CA). The spore wall was disrupted by agitation in a Mini-Bead Beater (BioSpec Products, Incorporation, Bartlesville, OK) for 1 min at

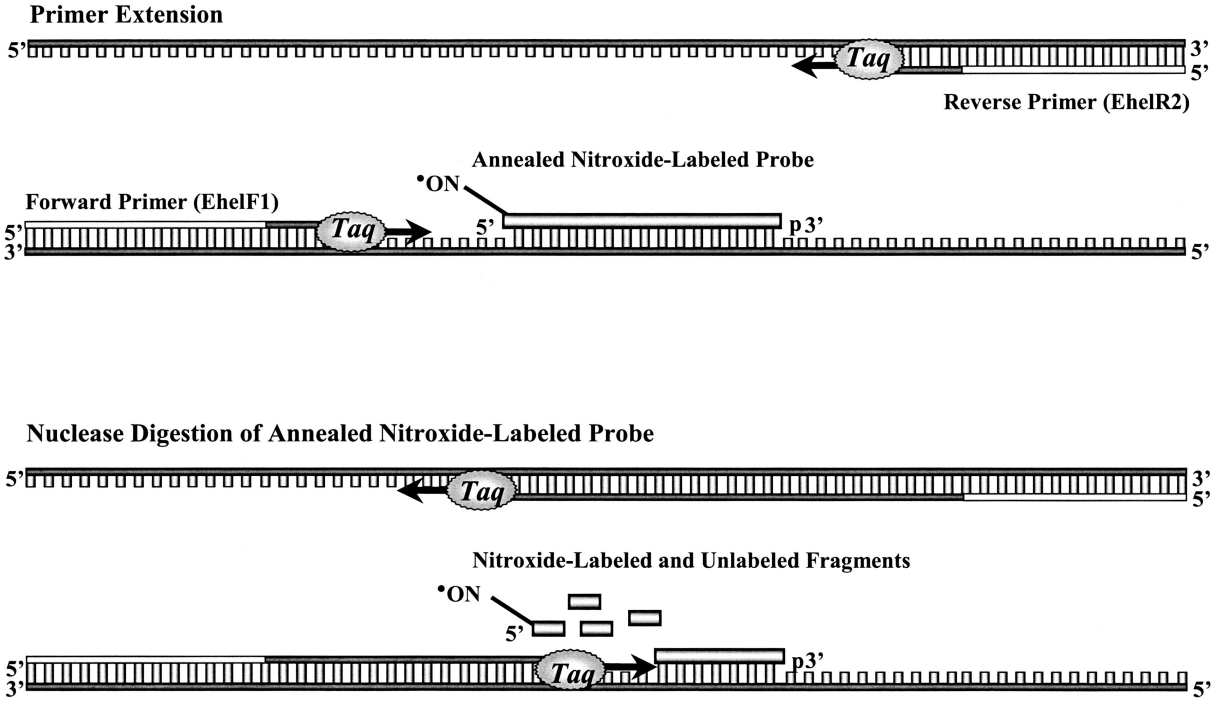

FIGURE 1 Scheme showing the formation of fast tumbling nitroxide-labeled fragments by nuclease digestion of annealed nitroxide-labeled oligonucleotide probes during the forward primer extension with *Taq* DNA polymerase. Additional annealed probes are digested in each PCR cycle.

maximum speed, the genomic DNA isolated with the DNeasy™ Tissue Kit, and the purified DNA stored at −20°C.

PCR Amplification of DNA

The PCR was done in a starting buffer of 10 mM Tris–HCl pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 0.176 μg/μl of bovine serum albumin Fraction V (Sigma–Aldrich Company), 6% (vol/vol) glycerol, 200 μM of each of the four deoxynucleoside triphosphates, 5–25 pmol of nitroxide-labeled probes, 10 pmol of each primer, 1.75 U of AmpliTaq Gold® DNA polymerase, and 5 μl of extracted microsporidial DNA template corresponding to either 1 or 10 spores in a volume of 50 μl using a PTC-200 thermal cycler (MJ Research Incorporation, Watertown, MA). After first heating the samples for 10 min at 95°C, 40 PCR cycles were performed (95°C for 0.25 min and 60°C for 1 min). Non-template controls (NTC) containing all the reagents except for DNA, and DNA isolated from *E. cuniculi* or *E. intestinalis* spores were used as controls.

Agarose Gel Analysis

PCR amplicons were analyzed by electrophoresis (1 h at 120 V) on a 3% high-resolution blend agarose gel (Amresco Incorporation, Solon, OH) containing ethidium bromide (1 μg/ml). The gels were visualized under UV, and digital images were obtained with a Kodak DC290 digital camera and Kodak 1D Image Analysis Software v 3.5.0 (Rochester, NY).

TABLE I Nucleic acid sequences and properties of the nitroxide-labeled probes ENSP, ENBP and EDUP

Probe/primer	Sequence (5′–3′)	Length (Nucleotides)	Target site location*	T_m (°C)†	A_{EPR} (cm/OD)
ENSP	Č‡CCTGTCCTTTGTACACACCGCCCp	24	1177–1200	70.4	56 ± 3
ENBP	Ť‡CCCTGTCCTTTGTACACACCGCCCp	25	1176–1200	71.5	42 ± 3
EDUP	Ť‡CCCTGTCCTTTGTACACACCGCCCp	25	1176–1200	71.5	21 ± 3
EhelF1	GAATGATTGAACAAGTTATTTTGAATGTG	29	1147–1175	60.3	
EhelR2	AACACGAAAGACTCAGACCTCTCA	24	1257–1234	64.4	

*Nucleotide positions based on the *Encephalitozoon hellem* 16S rRNA gene sequence (GenBank accession no. L39108).
† T_m, annealing temperature, calculated with MeltCalc software.[13]
‡ 1–3 See Fig. 3 for chemical structure of nitroxide-labeled nucleotides.

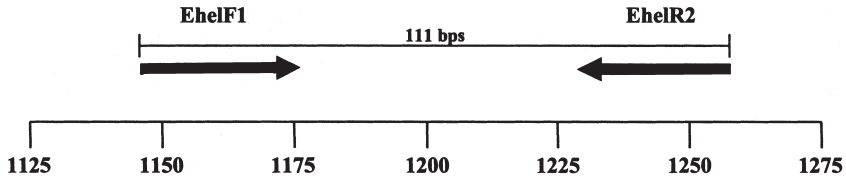

FIGURE 2 Positions of forward primer EhelF1 (29-mer) and reverse primer EhelR2 (24-mer) in the *E. hellem* 16S rRNA gene sequence (GenBank accession no. L39108), and length of the amplicons (111 bps) formed during PCR.

EPR Measurements

The CW-EPR spectra were digitally recorded either on a Varian E-104 or on a Bruker ESP 300 spectrometer in a TM_{110} cavity under non-saturating microwave power conditions using standard instrument parameters at room temperature. A chromium standard was fixed to the front of a small flat quartz cell to provide an up-field reference peak used as an external standard.[10] The EPR-specific activity (A_{EPR}) of the various probes was obtained as published,[1] and the presence of nuclease-digested nitroxide-labeled nucleic acid fragments was determined with the "free spin" test.[11]

RESULTS

Figure 1 is a schematic representation of the degradation of a nitroxide-labeled probe by the $5' \rightarrow 3'$ nuclease activity of *Taq* polymerase during the extension of the forward primer EhelF1 during a PCR cycle. The phosphate group at the 3′-end of the probe prevents its use as a primer for the polymerase activity of *Taq* polymerase. For each degraded probe a faster tumbling nitroxide-labeled fragment is formed as well as an amplicon of 111 base pairs (bps), the length of which is determined by the annealing sites of the primers, EhelF1 and EhelR2 (Table I) within the 16S rRNA gene of *E. hellem* (Fig. 2). The sequence of the primers EhelF1 and EhelR2 are specifically designed for *E. hellem* (GenBank accession no. L39108). The specific annealing of these primers will lead to probe fragmentation, while the same primers give three or more mismatches with the sequences of *E. cuniculi* (GenBank accession no. X98469) and *E. intestinalis* (GenBank accession no. U09929). The T_ms of the miss-matched systems is by at least 10°C below the values given in Table I for matched base pairing, and for that reason the primers EhelF1 and EhelR2 will not lead to probe fragmentation with *E. cuniculi* and *E. intestinalis* DNA. Thus, *E. cuniculi* and *E. intestinalis* DNA can serve as controls to establish the specificity of the *E. hellem* dependent probe fragmentation.

In Fig. 3 the chemical structure of the two five-membered nitroxide-labeled 5′-ends of the probes ENSP and ENBP are given as well as the six-membered nitroxide-labeled 5′-end of the probe EDUP that contains the spin-labeled DUMTA previously characterized in our laboratory.[12] The sequence for the probes is listed in Table I together with their target site location, T_m and A_{EPR} values. The T_ms of the 25-mers are about 1°C higher than the T_m of the 24-mer based on calculations using the MeltCalc software,[13] and the A_{EPR} are significantly higher for the five-membered nitroxides than the six-membered nitroxides as was already observed earlier for the DUMTA (containing six-membered nitroxide) and DUMPDA (containing five-membered nitroxide).[1]

Figure 4A shows the centerfield (h_0) and the high field (h_{-1}) line of the EPR spectra of the six-membered nitroxide in the spin-labeled nucleoside DUMTA and in the 25-mer EDUP. In both instances the line shape of h_0 and h_{-1} reflect fairly rapid tumbling nitroxides (correlation time $\approx 10^{-10}$ s) with the h_{-1}/h_0 ratios showing a significant difference for the two motional states. From extensive line shape simulations done on spin-labeled nucleic acid systems (for a review see Ref. [1] and references therein), it is also known that the h_{-1}/h_0 ratio can be used as a convenient ruler for evaluating changes in the nitroxide dynamics provided the spectra are in the motional narrowing region. It is possible to estimate an effective rotational correlation time τ from the signal heights h_0, h_{-1} and the peak-to-peak line width of h_0 by keeping in mind that the simple formalism assumes homogeneously broadening.[14] τ consists of fast motional components arising from internal motions (τ_i) usually expressed in terms of order parameter S[1] and from the slowly tumbling macromolecule (τ_{rb}). Assuming that the two motions are independent (motional independence is satisfied when the internal motion is significantly faster than the overall motion), the following relationship applies: $1/\tau = 1/\tau_i + 1/\tau_{rb}$, and we observed that h_{-1}/h_0 ratio increases with decreasing τ_{rb} for "flexi"-labeled nucleic acid systems.[15] Therefore, the h_{-1}/h_0 ratio of DUMTA should be greater than that of the 25-mer EDUP, which is indeed the case as shown in Fig. 4A. From Fig. 4A a h_{-1}/h_0 ratio of ≈ 0.85 is calculated for DUMTA, while the more slowly tumbling EDUP probe has a ratio of ≈ 0.56. Thus, the h_{-1}/h_0 ratio of "flexi" nitroxide-labeled nucleic acids serves as a molecular

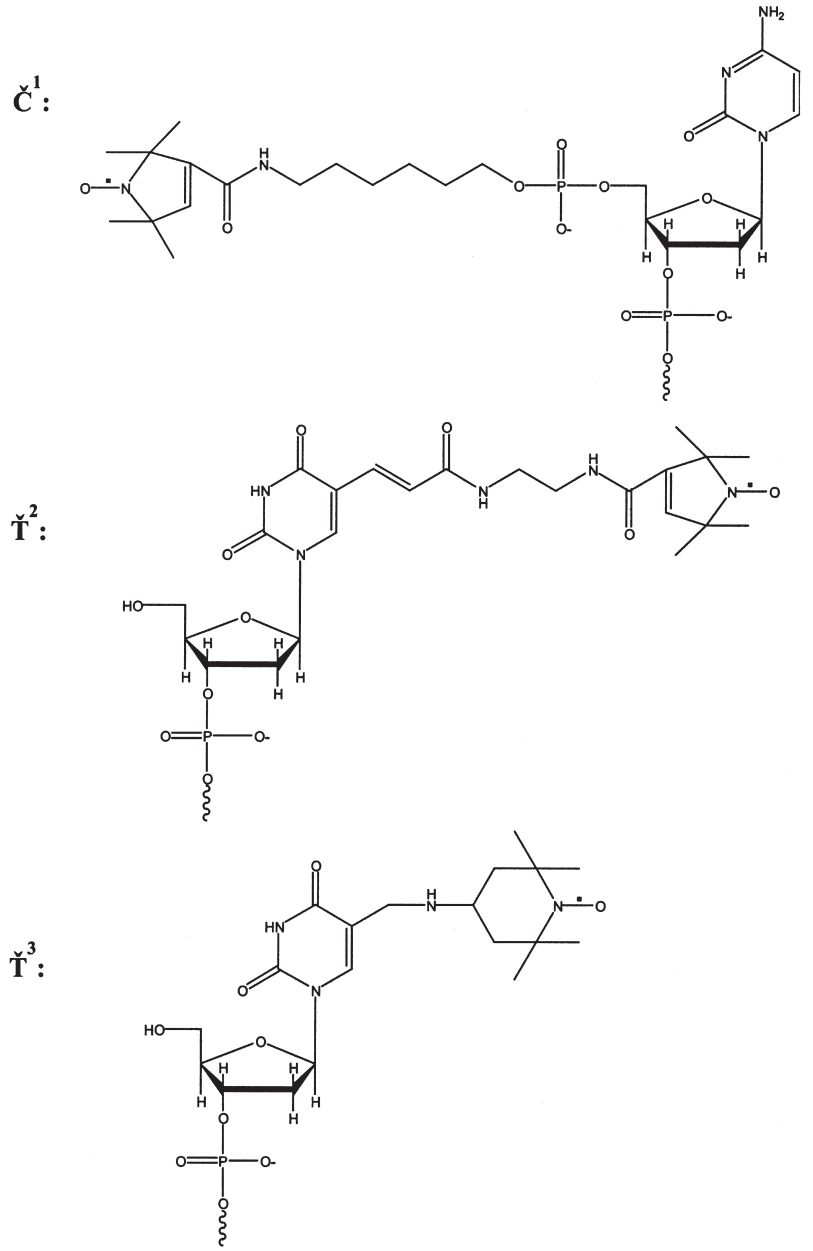

FIGURE 3 Chemical structure of the nitroxide-labeled nucleotides in the oligonucleotide probes: $\check{C}^1$: 5′-O-((2,5-dihydro-2,2,5,5-tetramethyl-1-oxyl-1*H*-pyrrole-3-carboxyl)aminohexyl)phosphoryl-2′-deoxycytidine in ENSP, $\check{T}^2$: 5-(*N*-((2,5-dihydro-2,2,5,5-tetramethyl-1-oxyl-1*H*-pyrrole-3-carboxyl)aminoethyl)-3-acrylimido)-2′-deoxyuridine in ENBP, and $\check{T}^3$: 5- ((2,2,6,6-tetramethyl-4-piperidyl-1-oxy)aminomethyl)-2′-deoxyuridine (DUMTA[1,12]) in EDUP. For the nucleic acid sequences of ENSP, ENBP and EDUP probes see Table I.

size sensor to conveniently detect the formation of nitroxide-labeled fragments.

Figure 4B shows representative EPR spectra of the center field (h_0) and high field (h_{-1}) line of the five-membered nitroxide in the 25-mer ENBP probe without DNA template and with DNA of a single *E. hellem* spore after 40 PCR cycles. The h_{-1}/h_0 ratio of the ENBP probe exposed to *E. hellem* DNA during PCR is significantly higher than the ratio measured in the absence of target DNA. Also, the h_{-1}/h_0 ratio of probe ENBP does not change after 40 PCR cycles in the absence of target DNA (data not shown), suggesting that the observed h_{-1}/h_0 ratio increase is not due to formation of nitroxide-labeled fragments by thermal degradation of the probe. In view of the signal to noise of the spectra no attempt has presently been made to quantify the fraction of degradation of the probe, although it is apparent from the significant increase in the h_{-1}/h_0, ratio that a large percentage of the ENBP probe is degraded with *E. hellem* DNA.

The generation of fast tumbling nitroxide-labeled fragments from the probe under sequence specific conditions has been validated with experiments shown in Fig. 5A that are based on forming complexes between positively charged poly-L-lysine and negatively charged probes. In the absence of nitroxide-labeled fragments most of the EPR signal

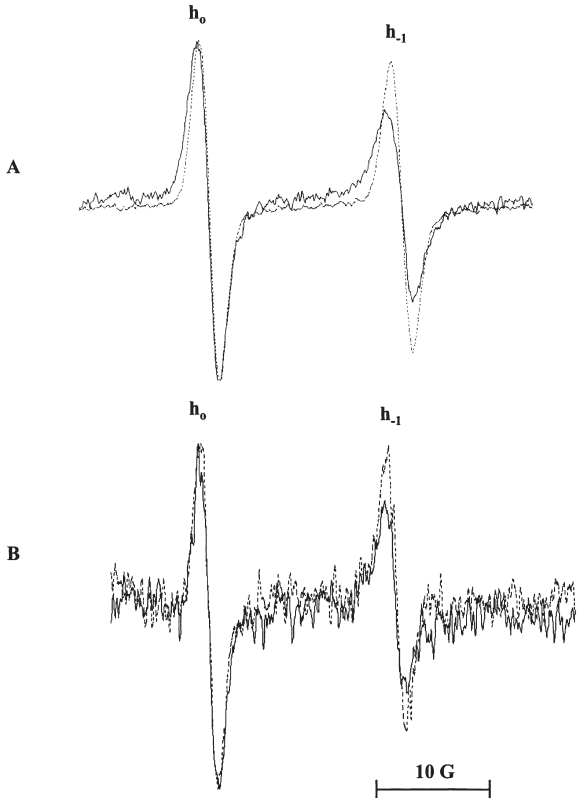

FIGURE 4 (A) h_0 and h_{-1} of the EPR spectra of 110 pmol of probe EDUP (solid line) and 120 pmol of the nucleotide DUMTA (broken line) in 200 µl buffer (0.1 M NaCl/0.01 M sodium cacodylate/0.01 M $MgCl_2$, pH 7.0), and (B) h_0 and h_{-1} of the EPR spectra of 9 pmol of the probe ENBP after 40 PCR cycles in 100 µl of 1:1 H_2O diluted PCR reaction mixture with DNA from a single *E. hellem* spore (broken line) and without DNA template (non-template control) (solid line). For comparison, the spectra are normalized with respect to h_0.

of the probe is quenched by poly-L-lysine, an effect that has been routinely used for the detection of contaminating, unbound nitroxides in spin-labeled nucleic acids ("free spin" test[11]). The intensity of the h_{-1} signal is particularly sensitive to mobility changes of nitroxides in the motional narrowing region (correlation time $\approx 10^{-10}$ s). An excess of poly-L-lysine complexed to the probe ENSP after 40 PCR cycles without DNA (non-template control) gives a strongly broadened h_{-1} signal with little intensity (Fig. 5A, spectrum a) due to the strongly hindered motion of the nitroxide in the probe complexed to poly-L-lysine. On the other hand, nitroxide-labeled fragments do not bind to poly-L-lysine and therefore their mobility remains unchanged. All the spectra in Fig. 5A include a Cr^{3+} signal used as an external standard[10] for quantifying h_{-1} intensity change of the probe as a function of the experimental settings as summarized in Table II. A visual inspection of Fig. 5A clearly reveals a sharpening and intensity increase of the h_{-1} signal in spectrum d and e. The signal increase, more pronounced for h_{-1} in spectrum e than d correlates well with the different amounts of *E. hellem* DNA used for the experiments. The DNA of *E. cuniculi* and *E. intestinalis*, for which the primers EhelF1 and EhelR2 are not complementary, gives a similar h_{-1} (spectra b and c) as the non-template control (NTC) (spectrum a). The nitroxide-labeled fragment formation from the probes listed in Table II follows the spectral pattern shown in Fig. 5A for ENSP. A negative sign in Table II corresponds to a ratio ≈ 1 for $h_{-1(\mathrm{DNA})}/h_{-1(\mathrm{NTC})}$, which indicates essentially no probe fragmentation, while the positive signs stand for ratios >1 with (+) signaling less fragmentation than (++). The data clearly show that the formation of faster tumbling nitroxide-labeled fragments increases with the amount of *E. hellem* DNA, and most importantly, probe fragmentation is not observed with DNA from *E. cuniculi* and *E. inestinalis*, since the *E. hellem* specific primers will not anneal to the latter two DNA sequences.

Further corroboration of the EPR data used to identify *E. hellem* DNA is presented with agarose gel electrophoresis results shown in Fig. 5B. The bands clearly show a relationship between the number of amplicons formed and probes degraded. From the Figs. 1 and 2 it is apparent that an amplicon of 111 bps is formed for each probe degraded. An aliquot of the PCR solutions used for measuring the EPR spectra shown in Fig. 5A was examined by agarose gel electrophoresis, where the lanes a–c correspond to samples with non-template control, *E. cuniculi* DNA and *E. intestinalis* DNA, respectively. No 111 bp bands are visible in the lanes a, b and c, and only lanes d and e containing *E. hellem* DNA show the expected amplicon bands with intensities correlating well with the EPR data.

DISCUSSION

It was suggested many years ago based on preliminary studies of nitroxides covalently bound to macromolecules that spin-labeled biomolecules have the potential for many applications.[14] Our laboratory selected nucleic acids as the biomolecule and together with others took advantage of the sensitivity of EPR spectroscopy to address mainly with SDSL DNA problems that are of biophysical nature. More recently, some laboratories have also started to work with SDSL RNA using non-perturbing nitroxides that are weakly coupled ("flexi" nitroxide) to the RNA matrix.[16,17] Even though the sensitivity of EPR spectroscopy is impressive, any practical application of this technique for nucleic acid identification has only now become a reality as shown with this preliminary study. The concept of using nitroxide-labeled nucleic acids to detect specific nucleic acid sequences is not new to this laboratory, since we reported already in 1975 that poly (A) tracts in mRNAs are detectable

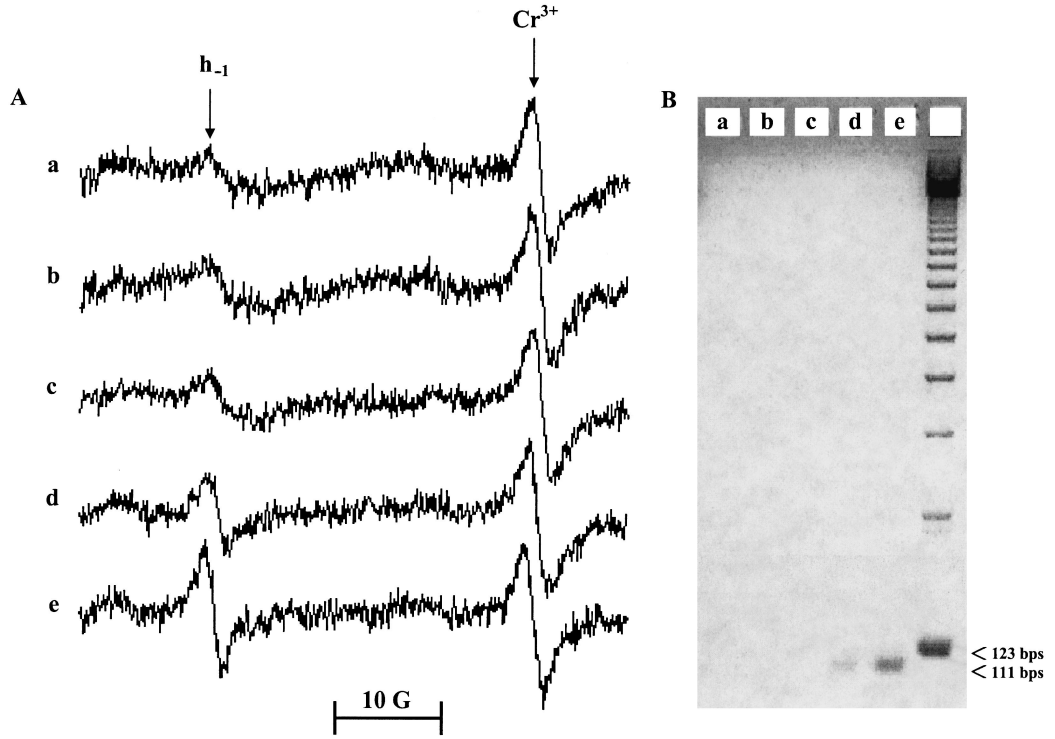

FIGURE 5 (A) EPR spectra of 25 pmol of the probe ENSP after 40 PCR cycles in 100 µl of 1:1 H_2O diluted PCR reaction mixture and addition of 4 nmol poly-L-lysine, and (B) agarose gel electrophoresis analysis of the PCR samples: a) without DNA template, b) with DNA from 10 *E. cuniculi* spores, c) with DNA from 10 *E. intestinalis* spores, d) with DNA from 1 *E. hellem* spore, and e) with DNA from 10 *E. hellem* spores.

with spin-labeled nucleic acids using EPR spectroscopy.[18] We also found that DNA sequences can be recognized in a loop-gap resonator with as little as 1 pmol of DNA.[12] In these previous studies, the detection was based on the reduced mobility of the nitroxide upon hybridization of the probe to the complementary sequence, which resulted in a characteristic broadening of the signal.[1]

In the present study, we take a novel approach and induce the formation of "free" spins under controlled conditions. The nitroxide-labeled nucleic acids are fragmented only in the presence of target DNA, and the resulting faster tumbling nitroxide-labeled fragments are readily observed by EPR. While this new strategy increases the detection sensitivity considerably, the breakthrough came by amplifying the probe fragmentation with PCR that makes it now possible to detect a single DNA genome with a low-end X-band EPR instrument. The PCR strategy was likely to succeed with "flexi" nitroxides, because we had previously shown that the polymerase activity of the *Taq* enzyme, an essential ingredient in PCR, is not affected with deoxynucleoside triphosphates weakly coupled to nitroxides.[19] The nuclease digestion results presented here show that "flexi" nitroxides are indeed also compatible with the exonuclease activity of the *Taq* enzyme.

Fluorescent technologies relying on the emission spectra of one reporter dye and the quenching properties of a second fluorescent dye (dual-label oligonucleotide reporter/ quencher systems) are currently used in DNA detection systems involving *Taq* polymerase. A good review of these approaches for clinical laboratories has recently been written.[20]

TABLE II Formation of nitroxide-labeled fragments with PCR-based digestion of nitroxide-labeled probes as determined by the "free spin test"

Probe	$h_{-1}{}^{*}{}_{(DNA)}$†/$h_{-1}{}^{*}{}_{(NTC)}$‡	$h_{-1}{}^{*}{}_{(DNA)}$¶/$h_{-1}{}^{*}{}_{(NTC)}$‡	$h_{-1}{}^{*}{}_{(DNA)}$§/$h_{-1}{}^{*}{}_{(NTC)}$‡	$h_{-1}{}^{*}{}_{(DNA)}$‖/$h_{-1}{}^{*}{}_{(NTC)}$‡
ENSP	−	−	+	++
ENBP	−	−	+	++
EDUP	−	−	+	++

* Normalized with respect to the Cr^{+3} standard.
† DNA of 10 *E. cuniculi* spores.
‡ Non-template control.
¶ DNA of 10 *E. intestinalis* spores.
§ DNA of 1 *E. hellem* spore.
‖ DNA of 10 *E. hellem* spores.

We found that EPR sensitive reporters offer at least the same sensitivity as the fluorescent/quencher systems. However, EPR based detection offers the unique advantage of requiring only one reporter group ("flexi" nitroxide) for each probe.

In the present study, we show the possibility of detecting one or more spores from the human pathogenic microsporidial species *E. hellem* by EPR while differentiating between DNA of *E. hellem* and that of the closely related *E. cuniculi* and *E. intestinalis*. Currently, histochemical stains such as the modified trichrome stain and fluorescent brighteners are used for routine clinical diagnosis of microsporidial infections.[21] However, these histochemical methods do not provide species-specific identification required for therapeutic strategies. Diagnostic assays utilizing monoclonal antibodies,[22] fluorescent *in situ* hybridization (FISH) probes[7] or PCR coupled with agarose-gel electrophoresis[22] have been shown to be specific for *E. hellem*, but all these approaches are based on complex processes not suitable for routine usage.

Finally, the EPR approach described for the detection of *E. hellem* DNA can be applied to other human-pathogenic species such as *Bacillus anthracis* (anthrax), West-Nile virus as well as to diagnose genetic diseases such as sickle-cell anemia, thalassamia, diabetes, and the like. In contrast to existing techniques based on fluorescence spectroscopy, EPR detection offers the advantage of no interference from turbidity of the solution, no interference from hydrophobic fluorophores and fluorescent background, and no tedious and expensive synthesis of probes. While the competing fluorescence-based methods rely on complex mechanisms requiring two labels for DNA identification, only one nitroxide label per probe is needed for EPR detection.

Acknowledgements

This work was in part supported by an appointment of (JDH) to the Postgraduate Research Participation Program administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Environmental Protection Agency. The authors would like to thank H.D. Alan Lindquist and Frank W. Schaefer, III for their interest in the project.

References

[1] Keyes, R.S. and Bobst, A.M. (1998) "Spin-labeled nucleic acids", In: Berliner, L.J., ed, Biological Magnetic Resonance (Plenum Press, New York), pp 283–338.
[2] Liang, Z., Freed, J.H., Keyes, R.S. and Bobst, A.M. (2000) "An electron spin resonance study of DNA dynamics using the slowly relaxing local structural model", *J. Phys. Chem. B* **104**, 5372–5381.
[3] Bobst, A.M. and Torrence, P.F. (1978) "Incorporation of spin-probes into polynucleotides by enzymatic polymerization", *Polymer* **19**, 115–117.
[4] Toppin, C.R., "Synthesis of analogues of uridine and 2′-deoxyuridine spin-labelled at positions 4 or 5 as substrates for RNA and DNA polymerizing enzymes" Ph.D. dissertation (University of Cincinnati, Cincinnati, OH).
[5] Okonogi, T.M., Reese, A.W., Alley, S.C., Hopkins, P.B. and Robinson, B.H. (1999) "Flexibility of duplex DNA on the submicrosecond timescale", *Biophys. J.* **77**, 3256–3276.
[6] Holland, P.M., Abramson, R.D., Watson, R. and Gelfand, D.H. (1991) "Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of *Thermus aquaticus* DNA polymerase", *Proc. Natl Acad. Sci. USA* **88**, 7276–7280.
[7] Hester, J.D., Lindquist, H.D., Bobst, A.M. and Schaefer, III, F.W. (2000) "Fluorescent *in situ* detection of *Encephalitozoon hellem* spores with a 6- carboxyfluorescein-labeled ribosomal RNA-targeted oligonucleotide probe", *J. Eukaryot. Microbiol.* **47**, 299–308.
[8] Pauly, G.T., Bobst, E.V., Bruckman, D. and Bobst, A.M. (1989) "Template-dependent incorporation of spin-labeled thymidine analogs' into viral DNA", *Helv. Chim. Acta* **72**, 110–116.
[9] Persichetti, R.A., Sinden, R.R., Duh, J.-L. and Bobst, A.M. (1991) "A comparative study of the phosphotriester versus the phosphoramidite approach to nitroxide labeled oligo dT", *Synth. Commun.* **21**, 1013–1019.
[10] Bobst, A.M., Ireland, J.C. and Bobst, E.V. (1984) "Nucleic acid binding affinity of fd gene 5 protein in the cooperative binding mode", *J. Biol. Chem.* **259**, 2130–2134.
[11] Bobst, A.M. (1979) "Applications of spin labeling to nucleic acids", In: Berliner, L.J., ed, Spin Labeling II: Theory and Applications (Academic Press, New York), pp 291–345.
[12] Strobel, O.K., Kryak, D.D., Bobst, E.V. and Bobst, A.M. (1991) "Preparation and characterization of spin-labeled oligonucleotides for DNA hybridization", *Bioconjug. Chem.* **2**, 89–95.
[13] Schutz, E. and von Ahsen, N. (1999) "Spreadsheet software for thermodynamic melting point prediction of oligonucleotide hybridization with and without mismatches", *Biotechniques* **27**, 1218–1228.
[14] Stone, T.J., Buckman, T., Nordio, P.L. and McConnell, H.M. (1965) "Spin-labeled biomolecules", *Proc. Natl. Acad. Sci. USA* **54**, 1010–1017.
[15] Keyes, R.S., Bobst, E.V., Cao, Y.Y. and Bobst, A.M. (1997) "Overall and internal dynamics of DNA as monitored by five-atom-tethered spin labels", *Biophys. J.* **72**, 282–290.
[16] Qin, P.Z., Butcher, S.E., Feigon, J. and Hubbell, W.L. (2001) "Quantitative analysis of the isolated GAAA tetraloop/receptor interaction in solution: a site-directed spin labeling study", *Biochemistry* **40**, 6929–6936.
[17] Macosko, J.C., Pio, M.S., Tinoco, I. and Shin, Jr, Y.K. (1999) "A novel 5 displacement spin-labeling technique for electron paramagnetic resonance spectroscopy of RNA", *RNA* **5**, 1158–1166.
[18] Bobst, A.M., Sinha, T.K. and Pan, Y.E. (1975) "Electron spin resonance for detecting polyadenylate tracts in RNA's", *Science* **188**, 153–155.
[19] Strobel, O.K., Keyes, R.S., Sinden, R.R. and Bobst, A.M. (1995) "Rigidity of a B-Z region incorporated into a plasmid as monitored by electron paramagnetic resonance", *Arch. Biochem. Biophys.* **324**, 357–366.
[20] Foy, C.A. and Parkes, H.C. (2001) "Emerging homogeneous DNA-based technologies in the clinical laboratory", *Clin. Chem.* **47**, 990–1000.
[21] Ignatius, R., Henschel, S., Liesenfeld, O., Mansmann, U., Schmidt, W., Koppe, S., Schneider, T., Heise, W., Futh, U., Riecken, E.O., Hahn, H. and Ullrich, R. (1997) "Comparative evaluation of modified trichrome and Uvitex 2B stains for detection of low numbers of microsporidial spores in stool specimens", *J. Clin. Microbiol.* **35**, 2266–2269.
[22] Visvesvara, G.S., Leitch, G.J., da Silva, A.J., Croppo, G.P., Moura, H., Wallace, S., Slemenda, S.B., Schwartz, D.A., Moss, D., Bryan, R.T. and Pieniazek, N.J. (1994) "Polyclonal and monoclonal antibody and PCR-amplified small-subunit rRNA identification of a microsporidian. Encephalitozoon hellem, isolated from an AIDS patient with disseminated infection", *J. Clin. Microbiol.* **32**, 2760–2768.