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Imaging of DNA sequences with chemiluminescence

(Maxam–Gilbert DNA sequencing/multiplex sequencing/enzyme substrates/alkaline phosphatase/biotinylated oligonucleotide probes)

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ABSTRACT **We have coupled a chemiluminescent detection method that uses an alkaline phosphatase label to the genomic DNA sequencing protocol of Church and Gilbert [Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* 81, 1991–1995]. Images of sequence ladders are obtained on x-ray film with exposure times of <30 min, as compared to 40 h required for a similar exposure with a ^{32}P-labeled oligomer. Chemically cleaved DNA from a sequencing gel is transferred to a nylon membrane, and specific sequence ladders are selected by hybridization to DNA oligonucleotides labeled with alkaline phosphatase or with biotin, leading directly or indirectly to deposition of enzyme. If a biotinylated probe is used, an incubation with avidin–alkaline phosphatase conjugate follows. The membrane is soaked in the chemiluminescent substrate (AMPPD) and is exposed to film. Dephosphorylation of AMPPD leads in a two-step pathway to a highly localized emission of visible light. The demonstrated shorter exposure times may improve the efficiency of a serial reprobing strategy such as the multiplex sequencing approach of Church and Kieffer-Higgins [Church, G. M. & Kieffer-Higgins, S. (1988) *Science* 240, 185–188].**

The ability to determine DNA nucleotide sequences rapidly and accurately has become increasingly important as efforts have commenced to determine the sequences of the large genomes of humans and other higher organisms. Both the chemical (1) and enzymatic (2) sequencing methods as originally conceived yield isotopically labeled sequence ladders (composed of nested DNA fragments terminating at an A, G, C, or T) that are imaged directly on x-ray film after gel electrophoresis. Efforts to improve the efficiency with which sequence information can be collected has focused on the detection of the nested DNA fragments generated by either method (3–9). Semiautomated approaches have been developed to detect the DNA fragments as they migrate through the gel, allowing direct computer storage and analysis of the sequence information. Two methods using this approach with multiemission fluorescent labels allow the mixtures from base-specific reactions to be loaded as a single gel lane (3, 4). Another, using ^{32}P, requires multiple lanes (10, 11).

Another approach is to perform the chemical sequencing reactions on a mixture of different DNA samples ligated into a series of plasmid vectors. After electrophoresis, the multiple sequence ladders are transferred to a membrane support and are accessed individually by using isotopically labeled oligomers specific for each plasmid vector. This method, called multiplex sequencing, can provide up to 150,000 nucleotides of sequence after 40 reprobings of a single membrane (12). The efficiency of this process depends on the number of reprobings and the time required to achieve them. A detection method with greater speed than ^{32}P autoradiography could enhance the efficiency of multiplex sequencing by affecting both parameters.

Chemiluminescence, the production of light by chemical reactions, has been explored as an alternative detection method to radioisotopes (13, 14). Most procedures are complicated by the number of reactants required to generate luminescent intermediates. Typically involved are the luminogenic substrate, one or more cofactors, an inorganic ion, an oxidant, and a catalyst. For example, the luminol reaction requires an oxidant, a catalyst, and alkali (15).

1,2-Dioxetanes are compounds that release light without the addition of any oxidants. These compounds contain the powerful oxidizing potential built into the molecules by the presence of inherently weak oxygen-oxygen bonds in highly strained four-membered rings (16). Decomposition of 1,2-dioxetanes is a highly exothermic process, releasing enough energy to allow the generation of products in electronically excited, emissive states (17, 18). Although many 1,2-dioxetanes have vanishingly short lifetimes at room temperature, substituents such as adamantyls have been found to confer remarkable stability (16, 19) and have permitted the synthesis of stable 1,2-dioxetanes that can be activated by enzymes (20–22). AMPPD [abbreviation derived from a nonsystematic name, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane, disodium salt] is a stable 1,2-dioxetane with a half-life of 1 year in aqueous solution (23). Dephosphorylation of AMPPD generates the unstable phenolate anion (AMP$^-$D), which decomposes and emits light (Fig. 1). Hence, AMPPD has been developed as a substrate for alkaline phosphatase and has been shown to be superior to the colorimetric substrates 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium in a number of applications (24, 25).

We describe the use of AMPPD to detect nucleotide sequence ladders on membranes generated by the genomic sequencing protocol of Church and Gilbert (26). DNA oligonucleotides labeled with alkaline phosphatase (27) or biotin are used as hybridization probes to select specific sequence ladders, leading directly or indirectly to the deposit of alkaline phosphatase at locations of interest. Addition of AMPPD leads to light emission detected by x-ray or black and white instant film. We demonstrate that the membrane can be stripped and reprobed with a different oligonucleotide to obtain other sequence ladders. The speed of this chemiluminescent detection method is superior to ^{32}P autoradiography.

MATERIALS AND METHODS

Materials. AMPPD is commercially available from Tropix, Inc. (Bedford, MA). Biodyne A nylon membrane (pore size, 0.45 μm) was purchased from Pall BioSupport Corp. (Glen Cove, NY); [γ-^{32}P]ATP was obtained from DuPont/NEN; polynucleotide kinase was from New England Biolabs; Ex-

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Abbreviations: AMPPD, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane; tPA, tissue plasminogen activator.

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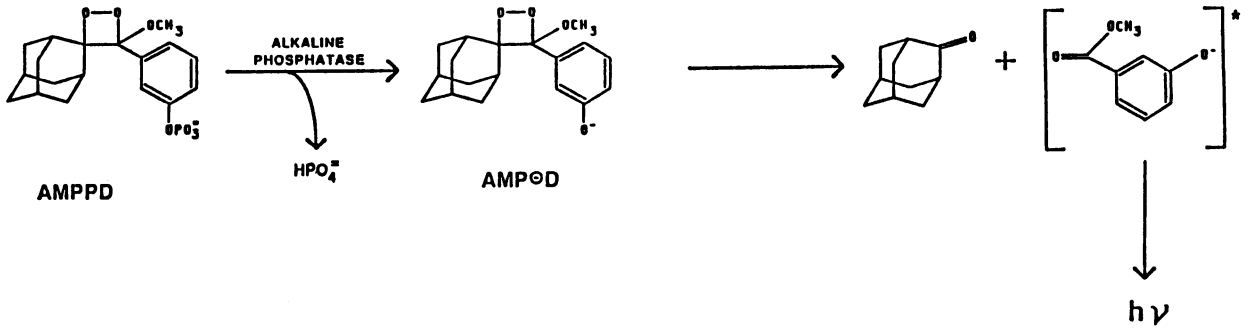

FIG. 1. Alkaline phosphatase-catalyzed chemiluminescence from AMPPD.

traAvidin-alkaline phosphatase was from Sigma; and Hammarsten grade casein was purchased from BDH.

Carbonate substrate buffer is 0.05 M sodium bicarbonate/carbonate, pH 9.5/1 mM $MgCl_2$. Phosphate-buffered saline (PBS) is 20 mM sodium phosphate, pH 7.2/150 mM NaCl. 1× SSC is 0.15 M NaCl/0.015 M sodium citrate. Diethanolamine substrate buffer is 0.1 M diethanolamine/1 mM $MgCl_2$/0.01% sodium azide, pH 10.0. Triton wash buffer is 1% Triton X-100/125 mM NaCl/50 mM Tris·HCl, pH 8.0. All buffers were prepared with distilled water.

Plasmid TPA55 DNA (a gift from Margaret Rosa, Biogen) contains a copy of the human tissue plasminogen activator (tPA) cDNA in pBR322. The biotinylated pBR322 oligonucleotide probe (5'-GCCGGAGCAAGAGCTACCAACTCTTTTTCCGAAGG-3') was purchased from Synthetic Genetics (San Diego). The KLR-230 oligonucleotide probe (5'-CTGGTATATCATCTGCGTTTTTTC-3'), which is specific for the tPA sequence, was synthesized on an Applied Biosystems 380A synthesizer and was either biotinylated by covalently attaching a biotinylated peptide to the 5' end (K.L.R. and R.L.C., unpublished data) or 5'-end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (28).

Plasmid pSQ214 contains a pBR322 insert cloned between two *Not* I sites. The alkaline phosphatase-labeled probe SNAP-B (a gift from Molecular Biosystems, Inc., San Diego, CA) hybridizes via 20 nucleotides of homology between one *Not* I site and the insert.

Sequencing Reactions, Hybridization, and Imaging. Sequence reactions and membrane preparations were performed essentially as described by Church and Gilbert (26). DNA for each of six Maxam–Gilbert chemical reactions consisted of 1 μg of plasmid DNA digested with restriction enzyme and Elutip (Schleicher and Schuell) purified. The TPA55 plasmid was digested with *Msp* I; pSQ214 was digested with *Not* I. Sonicated calf thymus DNA (4 μg) was added to each tube. The chemical cleavages consisted of the five originally described by Maxam and Gilbert—G, AG, AC, TC, and C (1)—and one other, T (29). One-seventh of each reaction mixture was loaded per lane onto a 0.4 mm TBE-gradient sequencing gel (60 cm long) (30). After 4 hr of electrophoresis, the DNA was electrotransferred to Pall Biodyne A (size, 0.45 μm) and UV treated (26). Membranes were dried to completion prior to a 5-min prehybridization in 1% crystalline grade bovine serum albumin/1 mM EDTA/0.5 M sodium phosphate, pH 7.2/7% SDS (26). The hybridization with biotinylated or ^{32}P-labeled oligonucleotide (0.8 nM) was performed for 12 hr at 45°C in the same buffer; enzyme-conjugated probe hybridization was performed for 2 hr at 45°C. After hybridization, the membranes were washed four times for 5 min at 45°C in 0.125 M NaCl/1 mM EDTA/1% SDS/0.04 M sodium phosphate, pH 7.2 (26). After this wash, the membrane handling differed depending on the probe used. All of the following steps were performed at room temperature. Membranes that were hybridized with ^{32}P-labeled oligomers were exposed to Kodak XAR-5 film at this stage. Membranes that were hybridized to biotinylated oligomers underwent the following additional incubations at room temperature. The membranes were first blocked with 0.2% casein in PBS for 1 hr and incubated for 30 min with a 1:5000 dilution of ExtraAvidin-alkaline phosphatase in the same buffer. The membranes were then washed two times with 0.2% casein in PBS for 5 min each, four times with 0.3% Tween 20 in PBS for 5 min each, and four times with carbonate substrate buffer for 1 min each. Finally, the membranes were incubated in 0.4 mM AMPPD in substrate buffer for 5 min, sandwiched between two layers of Saran-Wrap, and placed in contact with Kodak XAR or Polaroid type 803 instant black and white film for various times. The membrane hybridized to the enzyme-linked oligonucleotide was rinsed in Triton wash buffer briefly and then washed for 20 min in buffer. Two 1-min rinses in 1× SSC followed and, finally, there were two 1-min rinses in diethanolamine substrate buffer. The membrane was briefly incubated in 0.4 mM AMPPD in that same buffer, wrapped, and exposed.

Stripping and Reprobing. Membranes were stripped of hybridized probe and chemiluminescent material by treatment with 1% SDS in water. The SDS solution was heated to 90°C, poured over the membranes, allowed to cool to 70°C, and drained. The membranes were then hybridized with new probes, treated with ExtraAvidin-alkaline phosphatase, incubated with AMPPD, and imaged as described above.

Chemiluminescence Spectra and Kinetics. Chemiluminescence spectra were obtained in a Spex Fluorolog fluorimeter with the excitation source turned off. The spectrum of activated AMPPD in solution was obtained by adding 140 fmol of alkaline phosphatase oligonucleotide conjugate (27) to 3.0 ml of 0.4 mM AMPPD in AMPPD substrate buffer at room temperature. The spectrum of activated AMPPD on nylon was obtained by immobilizing 140 fmol of alkaline phosphatase oligonucleotide conjugate on Biodyne A membrane and incubating it in 0.4 mM AMPPD in substrate buffer.

The kinetics of light emission from alkaline phosphatase-activated AMPPD was measured in a Turner model 20E luminometer at 25°C. The kinetics of luminescence of activated AMPPD in aqueous solution was obtained by adding 14 fmol of an alkaline phosphatase oligonucleotide to 0.5 ml of 0.4 mM AMPPD in AMPPD substrate buffer. The kinetics of luminescence of activated AMPPD on nylon was obtained by immobilizing 14 fmol of alkaline phosphatase oligonucleotide conjugates on a 6-mm-diameter circle of Biodyne A membrane. The membrane was incubated for 5 min in 0.4 mM AMPPD in substrate buffer drained of excess buffer, heat sealed in a plastic bag, and placed directly on the photodetector window of the Turner luminometer to measure chemiluminescence.

RESULTS

Fig. 1 shows the two-step reaction initiated by enzyme-catalyzed dephosphorylation of AMPPD, which leads to the production of light. The AMP^-D produced upon dephospho-

rylation is moderately stable, and its half-life varies from 2 min to many hours, depending on its immediate environment (see below). As a result, $AMP^{-}D$ concentration increases until the rate of its decomposition is equal to the rate of its production from AMPPD. The chemiluminescence emission profile is in the form of a glow that persists for hours in solution (see Fig. 7*A*). On nylon, the kinetics are considerably slower, and the glow lasts for days (see Fig. 7*B*).

Fig. 3 shows two sequence patterns obtained from plasmid TPA55 (described in Fig. 2) with the chemiluminescent method. TPA55 DNA was digested with *Msp* I and subjected to six base-specific cleavage reactions. The cleavage products were resolved on sequencing gels and transferred to nylon membranes. Two identical membranes were hybridized with the two biotinylated oligomers shown in Fig. 2 followed by incubations with an avidin–alkaline phosphatase conjugate and AMPPD. One oligomer (pBR322) generates a sequence within the vector (Fig. 3*A*), while the other oligomer (KLR-230) generates a sequence within the tPA cDNA insert (Fig. 3*B*). Both images were obtained in 30 min. After 30 min or 24 hr, during which time $AMP^{-}D$ accumulation occurs, comparable exposures could be obtained in 10 min or 1 min, respectively. The amount of plasmid DNA loaded per lane (140 ng) is equivalent on a molar basis to the amount of individual plasmid DNA analyzed with the multiplex sequencing protocol (26). If each band represents 1/300th of the total DNA treated, then 0.14 fmol is present per band. Images can also be obtained by using Polaroid black and white instant film as shown in Fig. 4*B*.

To compare the chemiluminescent detection to the conventional method, which uses isotopically labeled probes, a third membrane was hybridized with a ^{32}P-labeled oligomer identical in sequence to the biotinylated tPA probe (KLR-230). The sequence pattern produced with the ^{32}P-labeled oligomer (3000 Ci/mmol; 1 Ci = 37 GBq) is shown in Fig. 4*A*. Since the exposure time required for this image was 40 hr, it can be estimated that the chemiluminescent method provides an 80-fold decrease in detection time compared to a ^{32}P-labeled oligomer with a specific activity of 3000 Ci/mmol.

To demonstrate that this detection system could also be adapted to multiplex sequencing, which requires serial reprobing of membranes, the experiment shown in Fig. 5 was performed. Fig. 5 shows the same membranes as Fig. 3 after the biotinylated probes have been removed by heating in 1% SDS and reprobed with the opposite probes to generate new sequences. The previous chemiluminescent sequence pattern has been completely removed by the simple wash used. Even the strongest signal on the membrane in Fig. 3*A*, representing the unreacted 190-base-pair fragment (indicated by the arrow), cannot be seen in the newly revealed sequence in Fig. 5*A*. Fig. 6 shows the pattern generated when an enzyme-conjugated oligonucleotide was used as hybridization probe.

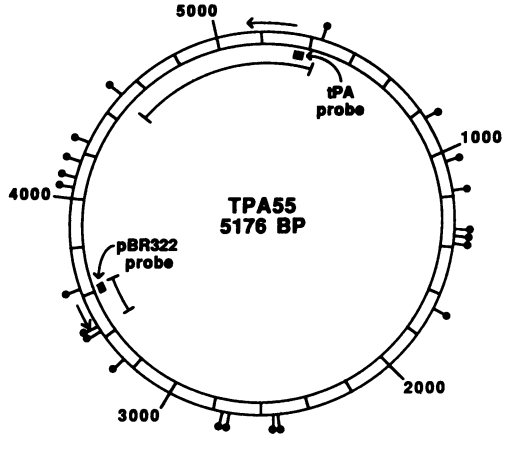

FIG. 2. Placement of *Msp* I sites and oligonucleotide probes on plasmid TPA55. The 21 *Msp* I restriction sites are indicated (●) and the location of the two oligonucleotide probes used in this study is also indicated. KLR-230 (24 bases) hybridizes to the tPA cDNA insert 41 nucleotides from the *Msp* I site at nucleotide 236 and generates the sequence shown in Figs. 3*B* and 5*A*. The pBR322-specific probe (35 bases) hybridizes close to the *Msp* I site at nucleotide 3645 and generates the sequence shown in Figs. 3*A* and 5*B*.

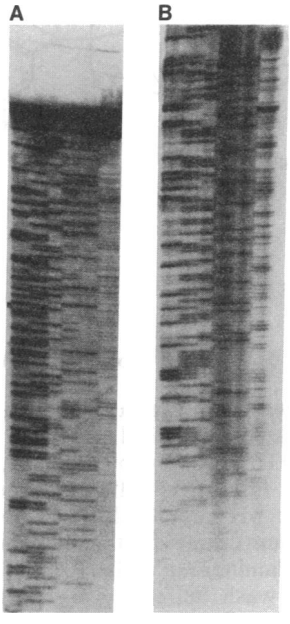

FIG. 3. Chemiluminescent detection of sequences imaged on x-ray film. Results are shown for membranes probed with the biotinylated pBR322 probe (*A*) and the biotinylated tPA probe KLR-230 (*B*). Exposures were for 30 min immediately after AMPPD incubation.

The nylon membrane has a significant effect on the emission characteristics of alkaline phosphatase-activated AMPPD. Fig. 7*A* shows the light emission from a pH 9.5 aqueous buffer solution of AMPPD after addition of alkaline phosphatase, while Fig. 7*B* shows the light emission after alkaline phosphatase activation of AMPPD on Biodyne A membrane. Although the variation in experimental parameters makes a direct comparison between the relative emission intensities in Fig. 7 impossible, the large difference in the rise times is significant. The results indicate that the rate of chemiluminescence on Biodyne A membrane is ≈2 orders of magnitude slower than in carbonate buffer solution. The chemiluminescence emission maximum is also affected by the nylon membrane. Fig. 8 shows that the emission spectrum of AMPPD activated on nylon membrane is hypsochromically shifted from the maximum of 477 nm in carbonate buffer to 460 nm. As discussed below, these results are consistent with $AMP^{-}D$ complexing or

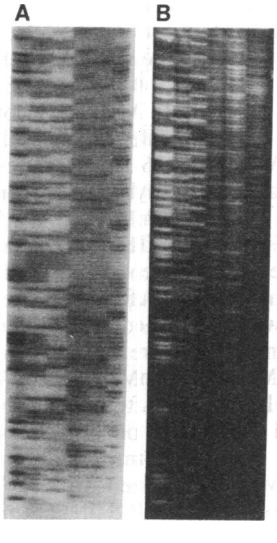

FIG. 4. Exposures of ^{32}P-labeled membrane imaged on x-ray film and chemiluminescent membrane imaged on instant black and white film. (*A*) Membrane probed with a ^{32}P-labeled KLR-230 oligonucleotide and exposed to x-ray film for 40 hr. (*B*) Membrane from Fig. 3*A* was exposed to Polaroid type 803 instant black and white film for 30 min to produce a positive image.

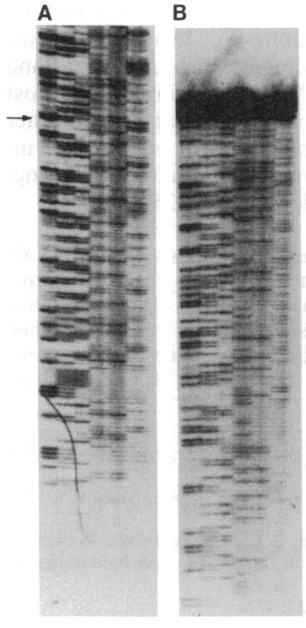

FIG. 5. Stripping, reprobing, and detection of sequences with chemiluminescence. The membranes shown in Fig. 3 were stripped as described in *Materials and Methods*. The membrane in Fig. 3*A* was reprobed with biotinylated KLR-230 probes (*A*), and the membrane in Fig. 3*B* was reprobed with the biotinylated pBR322 probe (*B*). Exposures were for 30 min immediately after AMPPD incubation. The stripping process is very efficient, as indicated by complete removal of the unreacted material (absent at the position shown by the arrow).

"binding" in the hydrophobic environment of the membrane. Complexation is presumably responsible for the sharpness of the sequencing bands observed in Figs. 3, 4*B*, 5, and 6.

DISCUSSION

We have coupled a chemiluminescent detection method to the genomic DNA sequencing protocol of Church and Gilbert (26). Images of sequencing ladders are obtained on x-ray film with exposure times of <30 min, as compared to 40 hr required for a similar exposure with a ^{32}P-labeled oligomer. DNA bands containing 0.14 fmol can easily be detected, generating images that are as sharp as ^{32}P images. Similar resolution with AMPPD has been observed by Beck *et al.* (25) after direct blotting electrophoresis of biotinylated sequence ladders. Their method cannot be applied to multiplex sequencing, however, since the transferred DNA is already biotinylated. Here we demonstrate the efficient stripping of biotinylated probes and AMPPD from membranes followed by reprobing to generate multiple sequences from a single membrane, a process that is not possible with colorimetric substrates since the precipitated product is difficult to remove (31, 32). Thus, the chemiluminescent detection method that we describe provides excellent resolution and sensitivity and can be adapted to multiplex sequencing.

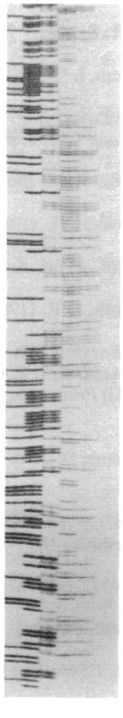

FIG. 6. Sequence detected with AMPPD chemiluminescent substrate and direct alkaline phosphatase-conjugated (SNAP) probe. This sequence was obtained in a 30-min exposure of pSQ214 DNA probed with SNAP-B.

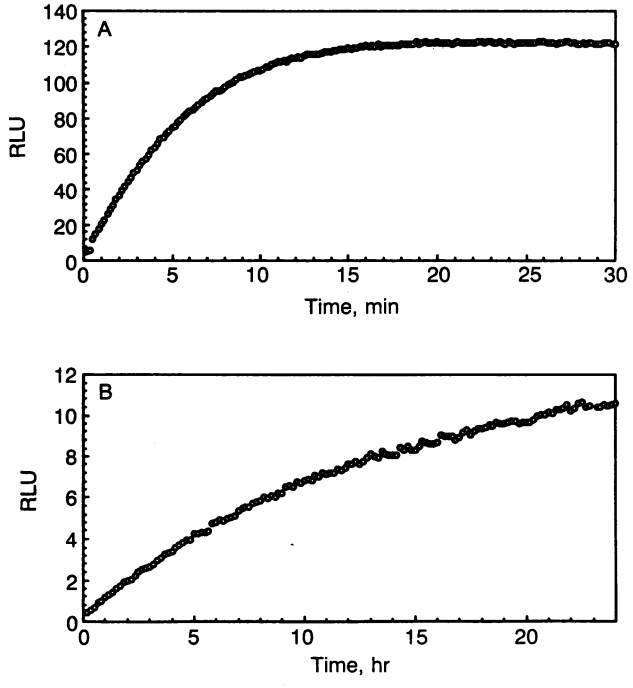

FIG. 7. Kinetics of chemiluminescence of alkaline phosphatase-catalyzed decomposition of AMPPD in 0.05 M bicarbonate/carbonate/1 mM $MgCl_2$, pH 9.5 (*A*), or on Biodyne A membrane (*B*). Luminescence rates are expressed as relative light units (RLU).

Chemiluminescent detection has the potential to increase the efficiency of multiplex sequencing since the nonradioactive probes and AMPPD are stable reagents and exposure times are considerably shorter. Although shorter exposures with ^{32}P can be achieved by using a higher specific activity probe (26), such a probe is expensive, has a short shelf life, and still fails to match the sensitivity of the chemiluminescence method. Intensifying screens can also decrease exposure times with ^{32}P, but they impair resolution of bands.

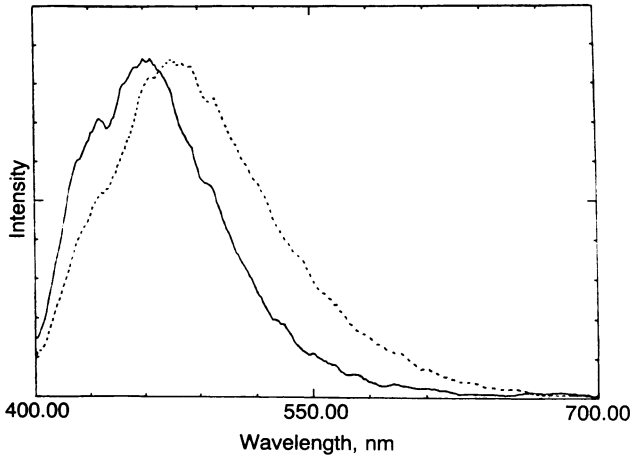

FIG. 8. Chemiluminescent spectra of the alkaline phosphatase-catalyzed decomposition of AMPPD in 0.05 M bicarbonate/carbonate/1 mM $MgCl_2$, pH 9.5 (---), or on Biodyne A membrane (—). Each spectrum represents the summation of five consecutive scans.

Chemiluminescent detection may offer an additional advantage over ^{32}P autoradiography, since the signal intensity is high enough for direct electronic imaging with charge-coupled devices, silicon-intensified targets, photon-counting cameras, or other sensitive scanning devices (33, 34), which would eliminate the film copy.

We have shown results with two different methods for targeting alkaline phosphatase to the membrane. Using the direct enzyme–DNA conjugate simplifies the procedure. By shortening the preimaging steps, which are not necessary with ^{32}P from ≈2 hr to 30 min, the direct enzyme conjugate minimizes this disadvantage of chemiluminescent detection. Using the direct enzyme conjugate also reduces the nonspecific membrane background that is observed in exposures longer than those shown here. Biotinylated probes have the advantages of being more widely available and yielding 2- to 3-fold darker images in a given exposure time. Recently, we have begun to investigate another technique for linking alkaline phosphatase to an oligonucleotide probe by a digoxigenin–antibody–alkaline phosphatase conjugate. These results will be reported separately.

Since AMP^-D is relatively long-lived, unlike the excited singlet emitter with a nanosecond lifetime (35), diffusion of AMP^-D could have resulted in high background and blurred bands. However, the sharp images of DNA bands shown in Figs. 3, 4*B*, 5, and 6 indicate that diffusion of AMP^-D does not occur and suggest that the anion may be complexed to the nylon. The effect of the nylon on the light-emission characteristics of alkaline phosphatase-activated AMPPD is consistent with the anion residing in a hydrophobic environment (36). The nylon membrane causes a shift in the chemiluminescence spectrum of the activated AMPPD from the maximum of 477 nm in aqueous buffer solution to 460 nm (Fig. 7), similar to the shift observed for other emitters in apolar solvents (37, 38). The hydrophobic environment stabilizes the dioxetane anion AMP^-D by complexing it in water-free domains. Association of AMP^-D with the membrane results in the sharp images of DNA bands, and also causes the kinetics of light emission to slow considerably (Fig. 7*B*). The slower kinetics may result because the complexed AMP^-D is restricted and its fragmentation is slower. The lower apparent pH environment of the nylon membrane may be further responsible for the slow kinetics of light emission by producing the protonated AMP^-D, which is stabilized. The pKa of this intermediate is ≈9 (23).

Serum albumin and other hydrophobic solutes improve the overall chemiluminescence intensity of activated AMPPD in aqueous solution and thereby act as "enhancers" to increase sensitivity and the speed of detection. This enhancement is achieved either by increasing the yield of chemiexcitation or by improving the quantum yield of emission from the excited molecules, or both. A similar enhancement of chemiluminescent yields apparently occurs on nylon, since signals with other membranes such as nitrocellulose are significantly lower but can be amplified by 2–3 orders of magnitude by the addition of enhancers (unpublished results). Consistent with this interpretation, enhancers only improve signals generated from nylon up to 5-fold (data not shown). This observation contradicts the assertion by Beck *et al.* (25) that enhancers can increase the luminescent yield of AMPPD on nylon by 300-fold. Nonetheless, it may be possible to design a new membrane superior to nylon and to generate the luminescent yields observed in nonaqueous solutions, which can be increased by 3 orders of magnitude over the yields observed in aqueous solution.

In summary, we have demonstrated that a chemiluminescent detection system for alkaline phosphatase can successfully be applied to the imaging of DNA sequences. The method, using a single-component chemiluminescent system, permits film exposure times much shorter than required for ^{32}P autoradiography of membrane-bound DNA without sacrificing sensitivity or resolution. We demonstrated this technology by using genomic sequencing of a plasmid and focused on streamlining multiplex sequencing as the most obvious application. This method could be applied to other techniques that generate membrane-bound sequences, including the original applications of genomic sequencing: protein footprints and methylation studies (26).

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