Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 3963–3965, July 1983
Biochemistry

Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination

[gel electrophoresis/deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate/dideoxynucleotide sequence analysis]

M. D. BIGGIN, T. J. GIBSON, AND G. F. HONG

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

Communicated by F. Sanger, April 4, 1983

ABSTRACT **Two methods for increasing the length of DNA sequence data that can be read off a polyacrylamide gel are described. We have developed a rapid way to pour a buffer concentration gradient gel that, by altering the vertical band separation on an autoradiograph, allows more sequence to be obtained from a gel. We also show that the use of deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate as the label incorporated in dideoxynucleotide sequence reactions increases the sharpness of the bands on an autoradiograph and so increases the resolution achieved.**

Both major rapid DNA sequence determination methods (1, 2) rely on thin denaturing polyacrylamide gels (3) to fractionate single-stranded DNA reaction products. To determine a sufficient length of sequence data from an experiment, it is usual to load aliquots from a reaction onto two or more gels run for various times. It would be advantageous to be able to obtain more sequence from each gel because this would allow either more total sequence to be obtained or fewer gels to be run per reaction. We describe two methods for increasing the amount of readable sequence per gel and demonstrate their effectiveness with the dideoxy sequence analysis technique.

One of the main limits on the length of sequence obtainable from an autoradiograph is the progressively poor separation of the bands corresponding to longer DNA molecules. Yet the spacing between the shorter DNA molecules is wider than is required for correct interpretation of the sequence pattern. It is possible, by use of a suitable gradient positioned only in the lower (anode) end of a gel, to selectively reduce the spacing between these shorter DNA molecules. By virtue of having traveled through a greater length of polyacrylamide gel, the higher molecular weight DNA molecules, lying above the gradient, will have an increased separation. We have found that an effective gradient consists of an increase in Tris/borate/EDTA (TBE) buffer concentration towards the bottom of the gel. A limit on the use of gradient gels has been the inconvenience and length of time needed for pouring such gels. We present a rapid technique, using simple apparatus, for pouring gradient gels, making their routine use for DNA sequence analysis realistic.

The principle by which a buffer gradient can be used to reduce the vertical band spacing on a polyacrylamide sequencing gel is that, as the buffer concentration increases progressively in the lower 10–15 cm of the gel, electrical resistance per cm down the gel decreases. This is because the buffer is the major charge carrier in the gel. Because the current is constant throughout the length of the gel, by Ohm's law the voltage drop per unit length will also decrease towards the anode. It is the potential difference across the gel that drives the polynucleotide migration, and so a fall in voltage drop per cm will cause a reduction in band migration rate. Thus the spacing between DNA molecules of n and $n + 1$ nucleotide residues can be reduced as they enter a gradient of increasing buffer concentration. Once such a gradient has been set up in a gel it is not expected to be significantly perturbed during electrophoresis, if diffusion is ignored. This is because the number of moving charge carriers is determined by the current, which is constant throughout the gel. Therefore the buffer ion migration caused by electrophoresis can be considered to be a process of serial ion displacement.

A further factor limiting the ability to read a sequence correctly, particularly from upper regions of a gel, is the sharpness or definition of individual bands. We show that incorporation of deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate (dATP[α-^{35}S]), instead of [α-^{32}P]dATP, as the radioactive label in dideoxy sequencing reactions, leads to an improved resolution of bands in a sequencing gel autoradiograph. dATP[α-^{35}S] is an analogue of dATP in which a sulfur atom replaces a nonbridge oxygen atom on the α-phosphate. The thio analogue is a substrate for *Escherichia coli* DNA polymerase I (4, 5). Where the radioisotope ^{35}S is used, the thio analogue provides a means of labeling DNA synthesized *in vitro*. The decay characteristics of ^{35}S are well suited to use in the dideoxy sequencing method.

MATERIALS AND METHODS

Gels. Gels are set between 20 cm × 50 cm glass plates separated by 0.35 mm Plasticard strips (Slaters, Matlock Bath, England). The loading wells are 3 mm wide and set 1.5 mm apart and are formed by using Plasticard combs. The various acrylamide gel solutions described all contain 6% acrylamide (19:1 acrylamide/bisacrylamide) and 460 g of urea per liter. The 10× TBE buffer is 108 g of Tris, 55 g of boric acid, and 9.3 g of EDTA (as disodium salt) per liter. Acrylamide gel mixes are made with 0.5×, 1.0×, and 5.0× TBE. The 5.0× TBE gel mix also contains 10% sucrose and bromophenol blue at 50 mg/liter. Gel mixes are not degassed.

Polymerization of acrylamide is initiated by addition of 1.5 μl of 25% ammonium persulfate stock solution and 1.5 μl of *N,N,N′,N′*-tetramethylethylenediamine per ml of gel mix. Directly after addition of polymerizing agents, gradient gels are poured in the following manner (standard gels being poured as in ref. 3). First, 35 ml of 0.5× TBE gel mix is drawn into a 50-ml disposable syringe and set aside for later use. A 25-ml graduated glass pipette is used to draw up 6 ml of 0.5× TBE gel mix by using a rubber Pro-pipette (Hoslab, London), followed by gently sucking up 6 ml of 5.0× TBE gel mix into the same pipette. As judged by the bromophenol blue, there is now a

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TBE, Tris base/boric acid/EDTA electrophoresis buffer; dATP[α-^{35}S], deoxyadenosine 5′-(α-[^{35}S]thio)triphosphate.

two-phase solution separated by a diffuse boundary. This is mixed slightly by causing one or two bubbles to run up the pipette by using a short sucking action of the Pro-pipette.

Pour the gels by running this crude gradient as a 5-cm broad stream down one side of a gel mold, much as when pouring a normal thin gel. When the pipette is almost empty, lower gel plates to a nearly horizontal position (e.g., supported on a 1-inch-diameter cork) to arrest flow of gel mix. Take the prefilled syringe and continue pouring the gel. The blue dye serves as a visual indication of how the buffer concentration gradient is forming in the lower 10–15 cm of the gel. The major problem in producing an even gradient across the gel is the tendency of the high concentration TBE gel mix at the bottom of the gel to flow back up the gel plates on the opposite side to the pouring side. This can be countered by gradually moving the point of addition of the 0.5× TBE gel mix towards the center of the gel plates. The presence of sucrose in the concentrated TBE gel mix also aids production of an even gradient across the width of the gel. However, with experience it is possible to omit the sucrose.

The buffer in the electrode tanks is 1× TBE for all gels. Gels are run at 28 mA until the bromophenol blue dye present in the formamide loading buffers has run off the gel for 10–15 min: this is usually about 3 hr for a gradient gel or 2 hr for a standard gel. After separation of the plates the gel is fixed in 1 liter of 10% acetic acid and 10% methanol (vol/vol) for 10–15 min. (The methanol prevents the bottom of the gels from swelling due to the sucrose, and the fixing time allows the urea to leach out of the gel, which improves the drying down.) Gels are then transferred to Whatman 3MM chromatography paper, covered with Saran Wrap (Dow) and the top ≈6 cm, including the wells, is trimmed off. Gels are dried down (6) for 15 min on a gel drier at 80°C, the Saran Wrap is removed, and the gels are then autoradiographed overnight. The drying down of gels and removal of Saran Wrap is particularly important for subsequent autoradiography of ^{35}S-labeled reaction products.

Reactions Procedure. The dideoxy sequence reactions (1, 7) are carried out in this laboratory in the following modified form. For each sequencing reaction the phage M13 template prepared as in ref. 8 is annealed with synthetic primer (9) in a 1.5-ml Sarstedt tube containing: 1.5 μl of primer at 0.1 pmol/μl, 1.5 μl of 100 mM Tris·HCl at pH 8.0, 50 mM $MgCl_2$, 3.0 μl H_2O, 5.0 μl of template DNA in 10 mM Tris·HCl/0.1 mM EDTA at pH 8.0. The sample is placed in a water bath at 75°C and the bath is allowed to cool for 0.5–1 hr, or alternatively the sample is put into an oven set at 60°C for 1 hr.

Synthesis reactions are carried out in four 1.5-ml capless Sarstedt tubes supported in a centrifuge rack (Eppendorf). Two microliters of annealing mixture is dispensed to the sides of each of these tubes. Four 1-μl aliquots of [α-^{32}P]dATP (approximately 400 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) are dried down in silane-treated glass tubes and resuspended in 2 μl of dideoxy T, C, G, or A nucleotide mixes (7), which are then dispensed into the reaction tubes. *E. coli* DNA polymerase I, large fragment (Boehringer Mannheim) is diluted to 1 unit in 8 μl with ice-cold 10 mM Tris·HCl, pH 8.0, 2-μl aliquots of which are quickly dispensed into the four tubes. Reactions start when the racks of tubes are centrifuged briefly at 2,000 rpm (Centra 3 centrifuge, International Equipment, Dunstable, England). After 15 min a chase of 2 μl of 0.5 mM dNTPs is added to each tube in the same manner. A further 15 min later reactions are stopped by addition of 4 μl of formamide dye mix to each tube. Reaction tubes are placed in a boiling water bath for 2.5 min just prior to loading 1 μl of samples onto the gels. Routinely, many sequencing reactions are done simultaneously and rapid dispensing of the nucleotides, enzyme, and dye mix is achieved by use of a multiple dispenser (PB 600-1, Hamilton, Bonacluz, Switzerland) equipped with silane-treated disposable tips. The removal of NaCl from the reaction conditions has been found to reduce markedly certain occurrences of the "pile-up" artefact associated with some runs of G residues on the template strand (unpublished data).

The substitution of dATP[α-^{35}S] (NEG-034H from New England Nuclear or a gift from Amersham), at a specific activity of 400–600 Ci/mmol, in the sequence reactions can be done with no additional changes to the protocol, as for Fig. 1. However, the following adjustments are now made: (*i*) Anneal with a mixture of 2 μl of primer (0.1 pmol/μl), 1.5 μl of 100 mM Tris·HCl, pH 8.0/50 mM $MgCl_2$, and 7 μl of template DNA. (*ii*) Use 1.5 units of enzyme to compensate for the extra annealed templates. (Note that this adjustment may apply only to some batches of polymerase.) (*iii*) Add chase mixture at 20 min and dyes at 35 min after the start of the reaction. (*iv*) Load 2 μl of samples onto the gel. (*v*) Halve the concentration of dideoxy ATP in the A reactions.

The purpose of these changes is to shorten the exposure time of the film during autoradiography and to eliminate an artefact band (corresponding to an A-1 position in a sequence) that sometimes occurs because of the slightly reduced rate of incorporation of dATP[α-^{35}S] into the DNA chain by DNA polymerase I. Dithiothreitol may be essential for the stability of dATP[α-^{35}S] during storage and in the reactions (unpublished data), but because it is included in commercial samples it should not be necessary to add extra.

RESULTS AND DISCUSSION

The left and center sequencing ladders in Fig. 1 demonstrate the different mobilities of DNA molecules from identical sequencing reactions when run on a TBE buffer gradient gel as compared with a normal thin gel. Below about 100 bases of insert sequence there is a relative condensing of the DNA sequence pattern on the gradient gel, which clearly demonstrates the action of the TBE concentration gradient. Above this position the band separation on the gradient gel is wider, due to the greater length of polyacrylamide gel that these bands have migrated through, when compared with equivalent bands on the standard gel autoradiograph. By using this gradient gel system one can acquire more sequence data from a single gel. The length of sequence determined from an autoradiograph is in part dependent on the quality of data one is prepared to accept. However, it would be usual to read approximately 200 bases of insert sequence from a standard 50-cm sequencing gel, such as the gel on the left of the figure. If band separation is the only criterion for the amount of data obtained from a gel, one can obtain about 90 more bases of sequence from the adjacent gradient gel because, at 290 bases above the M13 vector cloning site, the vertical band separation on the gradient gel is comparable to that of band separation at 200 bases on the standard gel.

The method of pouring the gel leads inevitably to a degree ofariation between gels. These differences between gels take the form of altered band spacing at comparable points up the gel, and sometimes curving of the lanes as shown in the figure. Neither of these problems has been found to be severe enough to interfere with the correct reading of a sequence, except in the most extreme cases, and these gels are now used routinely in this laboratory.

The volumes of the two different TBE concentration gel solutions and the relative differences in buffer concentration used in the pipette to form the gradient have been arrived at empirically to give a routinely effective gradient. For a shorter gel, 40 cm, for example, we have found a gradient of 0.5× TBE (4 ml) to 2.5× TBE (6 ml) to be more suitable because a 10-fold

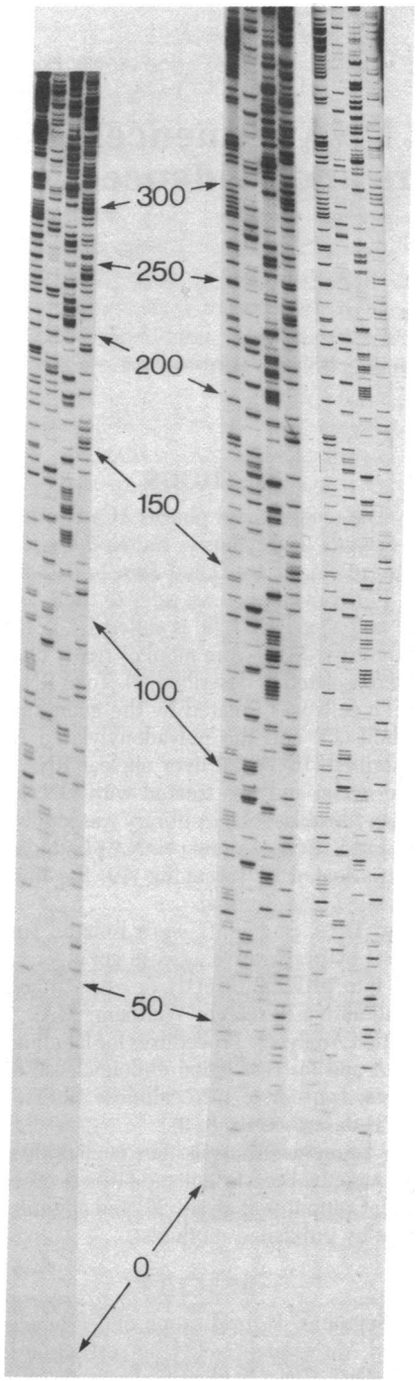

FIG. 1. Autoradiographs of standard sequencing gel (*Left*), [^{32}P]-dATP reactions on a TBE gradient gel (*Center*), and dATP[α-^{35}S] reactions run adjacent on the same gradient gel (*Right*). The same template DNA was used in all cases: an M13mp8 (10) *Sma* I-cut vector with a sonication fragment (11) insert derived from a cloned Epstein–Barr virus restriction fragment. The reaction conditions used were identical apart from substitution of dATP[α-^{35}S] for [α-^{32}P]dATP, at the same molecular concentration, in the *Right* sequence. Gels were run to give a similar short length of vector sequence remaining on the gel. The numbering commences at the start of the insert sequence. (Note that the actual length of DNA molecules is 48 nucleotides longer than numbered.) The lane order from left to right is T, C, G, A. The two ^{32}P-labeled sequences were autoradiographed overnight (14–16 hr) and the ^{35}S-labeled reaction products for about 30 hr.

concentration difference frequently leads to too severe condensing and distortion of the sequence pattern at the base of a 40-cm gel. The gel formed by this quick pour method is also rather imprecise in that the shape of the gradient cannot be calculated.

The use of dATP[α-^{35}S] as opposed to [α-^{32}P]dATP label in DNA sequencing reactions has three apparent advantages. (*i*) The major one is the very sharp definition of bands given by ^{35}S on a sequencing gel autoradiograph, which is a consequence of the short path length of the β particles emitted by ^{35}S. The strong emission of ^{32}P creates bands that are both larger (the size increasing with time of exposure) and more diffuse in appearance. These effects may be seen in the figure. Therefore, by using dATP[α-^{35}S], a sequencing gel can be read farther and with more confidence in the data obtained in the upper part of a gel. (*ii*) The weak emission of ^{35}S reduces the incident radiation dose received during handling of the labeled materials. (*iii*) The reaction products can be stored at −20°C for periods of a week or more before use in electrophoresis with minimal damage to the labeled DNA molecules (data not shown). Whereas decay of an incorporated atom of ^{32}P will break the sugar-phosphate backbone, this does not usually seem to be the case for decay of an incorporated ^{35}S atom. After storage of ^{35}S-labeled reaction products some faint artefact bands corresponding to A-1 positions can appear in the sequences, as predicted if radiolytic breakage occurs. The sulfur decays to a chlorine atom and the subsequent chemical readjustment and fate of the DNA chain may depend on the local chemical environment.

The basic disadvantage presented by ^{35}S is the longer exposure time required. In our experience the exposure time required for reaction samples of equivalent specific activity was 1.5 or 2 times longer. This can be circumvented by the adjustments to the reaction protocol given above or, alternatively, by using a sensitive x-ray film such as Kodak XAR-5. We routinely autoradiograph the gels for 16 hr with Fuji x-ray film.

The two methods described are simple ways of increasing the rate of sequence analysis in that they add little time to that required for determining the sequences of a given number of clones, need no elaborate equipment, and increase the amount of useful data per gel. The use of these improvements allows an average of between 260 and 300 nucleotides of sequence to be read with confidence from a single 50-cm gel for each clone sequenced, which is a significant increase over that obtained before. Previously our practice had been to load the products of a single sequencing reaction onto two gels, which were then run for different periods of time, usually for 2 and 4 hr. However, given the increase in data obtainable from a buffer gradient gel, we have found it more efficient when using the sonication "shotgun" cloning strategy (10, 11) to run samples on a single gel. The number of gels it is possible to run is a rate-limiting factor and so the above approach allows more M13 clones to be analyzed each day. We now normally determine the sequences of 28 clones per day, running samples on one gel only (seven samples per gel).

We particularly thank A. T. Bankier and B. G. Barrell for their help and advice.

1. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
2. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
3. Sanger, F. & Coulson, A. R. (1978) *FEBS Lett.* **87**, 107–110.
4. Vosberg, H. P. & Eckstein, F. (1977) *Biochemistry* **16**, 3633–3640.
5. Brody, R. S. & Frey, P. A. (1981) *Biochemistry* **20**, 1245–1252.
6. Garoff, H. & Ansorge, W. (1981) *Anal. Biochem.* **115**, 450–457.
7. Walker, J. & Gay, N. J. (1983) *Methods Enzymol.*, in press.
8. Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. A. (1980) *J. Mol. Biol.* **143**, 161–178.
9. Duckworth, M. L., Gait, M. J., Goelet, P., Hong, G. F., Singh, M. & Titmus, R. C. (1981) *Nucleic Acids Res.* **9**, 1691–1706.
10. Messing, J. & Vieira, J. (1982) *Gene* **19**, 269–276.
11. Deininger, P. (1983) *Anal. Biochem.* **129**, 216–223.