tation into a target protein for megaprimer that is longer than 300 bp. To our knowledge, this is the first work with very high efficiency (approaching 100%) for site-directed mutagenesis PCR using megaprimer that is 800 bp in length. This method will be of particular utility for producing the mutations in long target template with long megaprimer.

REFERENCES

1. **Barik, S. and M.S. Galinski.** 1991. "Megaprimer" method of PCR: increased template concentration improves yield. BioTechniques *10*:489-490.
2. **Colosimo, A., Z. Xu, G. Novelli, B. Dallapiccola, and D.C. Gruenert.** 1999. Simple version of "megaprimer" PCR for site-directed mutagenesis. BioTechniques *26*:870-873.
3. **Datta, A.K.** 1995. Efficient amplification using "megaprimer" by asymmetric polymerase chain reaction. Nucleic Acids Res. *23*:4530-4531.
4. **Giebel, L.B. and R.A. Spritz.** 1990. Site-directed mutagenesis using a double-stranded DNA fragment as a PCR primer. Nucleic Acids Res. *25*:4947.
5. **Ling, M.M. and B.H. Robinson.** 1997. Approaches to DNA mutagenesis: an overview. Anal. Biochem. *254*:157-178.
6. **Sarkar, G. and S.S. Sommer.** 1990. The "megaprimer" method of site-directed mutagenesis. BioTechniques *8*:404-407.
7. **Smith, A.M. and K.P. Klugman.** "Megaprimer" method of PCR-based mutagenesis: the concentration of megaprimer is a critical factor. BioTechniques *22*:438-442.

We are indebted to Dr. Melissa Brown (The Department of Biochemistry and Molecular Biology, The University of Queensland) for the kind gift of wild-type p53 *clones.* *Address correspondence to A/Prof. Ross Barnard, The Department of Biochemistry and Molecular Biology, The School of Molecular and Microbial Science, The University of Queensland, Brisbane, Queensland 4072, Australia. e-mail: rossbarnard@mailbox.uq.edu.au*

Received 15 July 2002; accepted 16 October 2002.

R. Lai, A. Bekessy, C.C. Chen, T. Walsh[1], and R. Barnard
The University of Queensland
[1]The Queensland University of Technology
Brisbane, Queensland, Australia

Reverse-Polarity PAGE for Examining DNA Binding Domain Phosphorylation

BioTechniques 34:56-59 (January 2003)

As interest in the signaling pathways impacting nuclear protein function increases, more investigators are examining transcription factor phosphorylation. Most phosphorylation events within proteins can be mapped using a variety of 1-D or 2-D phosphopeptide mapping approaches (2–4,6,8). However, these conventional approaches may not be optimal for examining phosphorylation events that occur within the DNA binding domains of transcription factors. Since DNA binding domains typically contain a high proportion of basic residues and most electrophoretic phosphopeptide mapping techniques separate molecules based on size and net negative charge, peptides containing a basic DNA binding domain usually migrate through conventional gels very poorly, if at all. An alternative method for examining the phosphorylation state of basic DNA binding domains would be significant, since post-translational modification of these domains has the potential of altering the interaction of transcription factors with target DNA and contributing to the regulation of the function of these proteins in vivo (5,7,9).

Here we describe reverse-polarity PAGE, a phosphopeptide mapping approach designed for examining phosphorylation events within the basic DNA binding domains of transcription factors. The principle of this technique is to perform PAGE with the anode and cathode leads reversed, thus allowing only peptides with a net positive charge to migrate through the gel. This approach addresses problems inherent to the detection of highly basic peptides with conventional PAGE by focusing on the resolution of positively charged peptides, rather than all peptides, from a protein digest. Since relatively few phosphopeptides from a typical protein digest are likely to have a net positive charge, the number of peptides detected by reverse-polarity PAGE is small and data interpretation is simplified.

Vertical slab gels ($0.75 \times 18 \times 16$ cm) for the Hoefer SE 600 electrophoresis apparatus are used for reverse-polarity PAGE. The resolving gel (19% acrylamide, 0.018% bis-acrylamide, 0.1% ammonium persulfate, and 10 µL TEMED) is prepared in 100 mM sodium phosphate buffer, pH 6.0, cast leaving approximately two inches of space for the stacking gel and overlaid with butanol until polymerization occurs. The butanol is removed with several washes of deionized water and 10 mL stacking gel (3% acrylamide, 0.125% bis-acylamide, 0.075% ammonium persulfate, and 10 µL TEMED) in 100 mM sodium phosphate buffer, pH 7.0, is added, followed by insertion of a 15-well comb. The electrophoresis apparatus is assembled, and 100 mM sodium phosphate buffer, pH 6.0, is used in the upper and lower chambers as running buffer. Samples are prepared by combining equal volumes of sample and loading buffer (1 M acetic acid, 6 M urea, 5% sucrose, and cytochrome C for color), and the current is applied to the loaded gels after reversing the anode and cathode leads. To prevent damage to the electrophoresis apparatus, it is important to ascertain whether the anode and cathode materials are compatible with reverse-polarity PAGE; platinum wire electrodes were used for all reverse-polarity PAGE experiments described. Samples are subjected to electrophoresis at 20 mA for the first 3 h. Once the positively charged peptides have moved into the resolving portion of the gel, the current can be increased to 40 mA without compromising peptide resolution. To reduce the effects of heat generated during electrophoresis, it is helpful to limit the voltage and/or current to 200 V and 40 mA, respectively, and to run the gels at 4°C. We also recommend that the buffer in the upper and lower reservoirs be changed every 6–8 h. The typical run time for the reverse-polarity PAGE performed in this report was approximately 18 h. However, the run time will vary with the properties of individual peptides and must be established empirically.

To optimize this technique, we chose to analyze peptides representing the DNA binding domains from the basic leucine zipper transcription factors, BATF (^{36}NRIAAQKSRQRQ^{47}) and

Table 1. Strategies to Liberate Transcription Factor DNA Binding Domains for Analysis by Reverse-Polarity PAGE

Transcription Factor	Class	Enzyme	No. Peptides[a]	No. Peptides (+NC)[b]
C/EBP b	bZIP[c]	Asp-N	11	2
		Glut-C	16	1
Oct 1	POU	Asp-N	15	1
		Glut-C	18	2
p53	unique	Asp-N	18	3
MyoD	bHLH[d]	Glut-C	16	3
SP1	Zn^{2+} finger	Asp-N	14	3
		Glut-C	21	3

[a]peptides ≥ MW 500 Da
[b]positive (+) net charge (NC) at pH 6.0
[c]basic leucine zipper
[d]basic helix-loop-helix

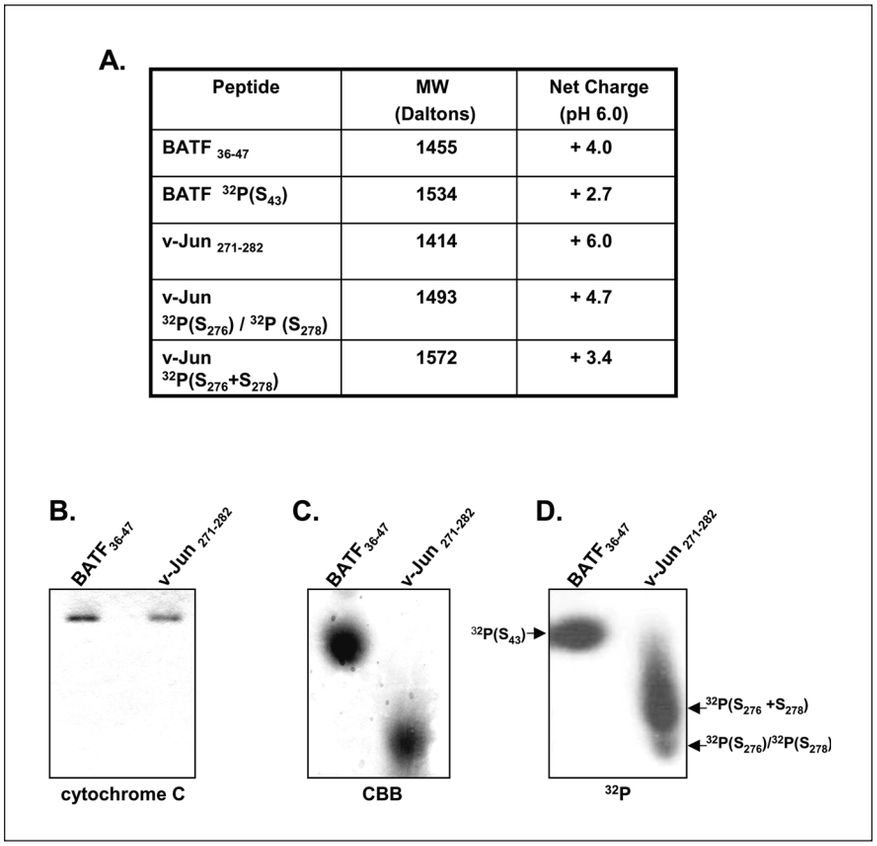
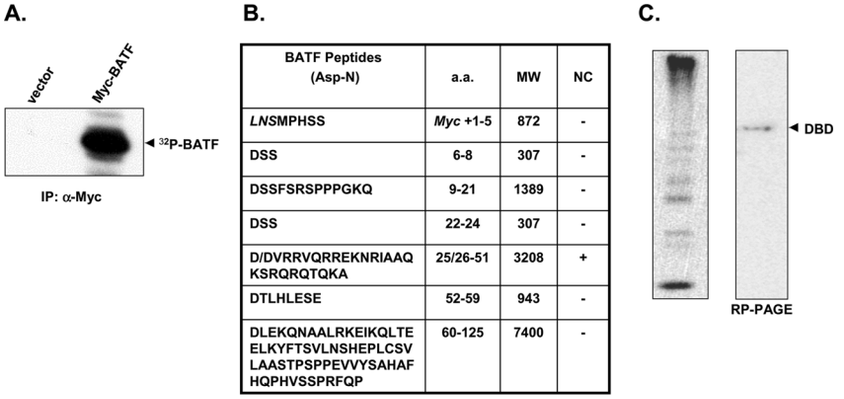


Figure 1. Reverse-polarity PAGE resolves positively charged phosphopeptides representing the BATF and v-Jun DNA binding domains. (A) The chart lists the molecular weight (MW) and charge (Net Charge) of the control and phosphorylated BATF and v-Jun peptides used for these experiments. Numbers refer to the amino acid positions in the human (BATF) or viral (v-Jun) proteins. Net charge of a peptide is determined as described previously (4), by summing the charges of the N and C termini plus the side chains of its amino acids at a given pH (6.0). (B) Migration of the cytochrome C indicator by reverse-polarity PAGE. (C) Coomassie Brilliant Blue (CBB) staining of unphosphorylated BATF and v-Jun peptides resolved by reverse-polarity PAGE. (D) Autoradiogram of BATF and v-Jun peptides phosphorylated in vitro by PKC-α and resolved by reverse-polarity PAGE. Arrows indicate migration of singly and doubly phosphorylated forms of the peptides.

v-Jun (^{271}NRIAASKSRKRK^{282}) (1,7). These peptides have similar molecular weights but different net charges at pH 6.0 (Figure 1A). Approximately 5 µg each unmodified peptide were resolved by reverse-polarity PAGE using the aforementioned methodology. The cytochrome C contained in the loading buffer remained visible throughout the run because of its positive charge and red pigmentation (Figure 1B). The unmodified peptides, once resolved, were detected by staining the gels with Coomassie Brilliant Blue® (Figure 1C). Even though the v-Jun and BATF peptides have similar molecular weights, they exhibit discrete mobilities by reverse-polarity PAGE due to a difference in net charge. The ability of reverse-polarity PAGE to separate peptides of similar molecular weight based on net charge is particularly useful when examining peptides with multiple phosphorylation events. To illustrate this point, the v-Jun and BATF peptides (1 µg each) were radiolabeled using [γ-^{32}P]ATP, purified protein kinase C-α (PanVera, Madison, WI, USA), and reaction conditions as described (7). Approximately 20 ng each peptide were resolved and visualized after exposing the dried gel to X-ray film (Figure 1D). The difference in mobility between the two peptides is similar to what was observed by Coomassie Brilliant Blue staining (Figure 1C). However, the v-Jun peptide resolves as two radiolabeled bands corresponding to singly and doubly phosphorylated forms. In contrast, the BATF peptide contains only one residue subject to phosphorylation and is visualized as a single radiolabeled band.

To demonstrate the utility of reverse-polarity PAGE for mapping in vivo phosphorylation events occurring within a basic DNA binding domain of a transcription factor, we examined the phosphorylation state of the BATF protein in mouse EL-4 thymoma cells. Briefly, 0.5×10^6 EL-4 cells were electroporated using a Bio-Rad Gene Pulser® II (310 mV, 950 µF; Bio-Rad Laboratories, Hercules, CA, USA) and 5 µg control vector (pCS2+MT) or vector expressing Myc-epitope tagged BATF (pCS2+MT-BATF). After a 5-h recovery, the cells were washed and resuspended in phosphate-free RPMI medium for 1 h, after which time 2 mCi radiolabeled inorgan-



BATF Peptides (Asp-N)	a.a.	MW	NC
*LNS*MPHSS	*Myc* +1-5	872	-
DSS	6-8	307	-
DSSFSRSPPPGKQ	9-21	1389	-
DSS	22-24	307	-
D/DVRRVQRREKNRIAAQ KSRQRQTQKA	25/26-51	3208	+
DTLHLESE	52-59	943	-
DLEKQNAALRKEIKQLTE ELKYFTSVLNSHEPLCSV LAASTPSPPEVVYSAHAF HQPHVSSPRFQP	60-125	7400	-

Figure 2. In vivo phosphorylation of the BATF DNA binding domain is detected by reverse-polarity PAGE. (A) Autoradiogram of immunoprecipitated, ^{32}P-labeled BATF phosphoprotein resolved by SDS-PAGE. (B) The molecular weight (MW) and net charge (NC) of the peptides predicted to be generated from the cleavage of Myc-epitope tagged BATF with Asp-N. The DNA binding domain [amino acids (a.a.) 26–51; MW 3208Da] is the sole peptide with a net positive charge. (C) Resolution of BATF phosphopeptides by forward-polarity PAGE (left) and reverse-polarity PAGE (right). The negatively charged phosphopeptides are resolved by forward polarity. The positively charged DNA binding domain (DBD) is resolved by reverse-polarity PAGE and appears as a phosphopeptide following autoradiography.

ic phosphorous ([^{32}P]H_3PO_4) were added for 5 h. The cells were washed to remove as much unincorporated label as possible and lysed in RIPA buffer. Supernatants were subjected to immunoprecipitation with 5 µg 9E10 mAb (Roche Applied Science, Indianapolis, IN, USA) and 20 µL protein A Sepharose (Amersham Biosciences, Piscataway, NJ, USA) for 1.5 h at 4°C. The immunopreciptates were washed five times in RIPA buffer plus protease inhibitors (Sigma, St. Louis, MO, USA), boiled in SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose as described previously (3), and visualized by autoradiography (Figure 2A). The filter strip containing phosphorylated BATF protein was incubated for 30 min at 37°C in 0.5% polyvinylpyrolidone-360 (Sigma) and 100 mM acetic acid, washed with water, and transferred to 300 µL of 50 mM NH_4HCO_3 for protease digestion. For RP-PAGE to yield meaningful information about specific regions of a protein (in this case, the DNA binding domain), it is important to select an appropriate method for protein fragmentation. Cleavage within the DNA binding domain should be avoided, since this may reduce the net positive charge of the peptide of interest. Thus, proteases that target basic residues, including trypsin, Arg-C, or Lys-C, are less desirable than proteases that target acidic residues (e.g., Asp-N or Glut-C). Consideration of other enzymes, or chemical cleavage, may be necessary, depending on the sequence of the protein and the basic DNA binding domain being studied. For the analysis of BATF DNA binding domain phosphorylation, we chose endoproteinase Asp-N (Sigma), since the BATF DNA binding domain is the only positively charged peptide predicted to be generated following cleavage (Figure 2B). Phosphorylated BATF was incubated with Asp-N for a total of 5 h at 37°C, adding 3 ng/µL of the enzyme at the start of the incubation and another 3 ng/µL at the 3-h timepoint to maximize digestion. After removal of the filter, water was added, and the ammonium salt was eliminated from the peptide solution by repeated lyophilization. The dried sample was resuspended in loading buffer and resolved using 40% alkaline PAGE (forward polarity) or reverse-polarity PAGE (Figure 2C). Resolution by forward-polarity PAGE reveals the existence of numerous phosphopeptides with net negative charges, while reverse-polarity PAGE resolves only one phosphopeptide representing the positively charged, basic DNA binding domain of BATF.

While experiments in this study have demonstrated the utility of reverse-polarity PAGE for the analysis of the v-Jun and BATF DNA binding domains, the technique should be useful for similar analyses on a broad spectrum of transcription factors. Table 1 provides examples of transcription factors possessing basic DNA binding domains that contain at least one potential site for phosphorylation and that can be liberated from their parent proteins as one of a few (≤3) positively charged peptides produced by Asp-N or Glut-C cleavage. The ability to separate these DNA binding domains-containing peptides from the remaining negatively charged peptides by reverse-polarity PAGE will greatly simplify experimental approaches to examine DNA binding domain phosphorylation and the potential role of DNA binding domain phosphorylation in modulating transcription factor function in response to specific cellular signaling events.

REFERENCES

1. **Dorsey, M.J., H-J. Tae, K.G. Sollenberger, N.T. Mascarenhas, L.M. Johansen, and E.J. Taparowsky.** 1995. BATF: a novel human bZIP protein that associates with members of the AP-1 transcription factor family. Oncogene *11*:2255-2265.
2. **Gatti, A. and J.A. Traugh.** 1999. A two-dimensional peptide gel electrophoresis system for phosphopeptide mapping and amino acid sequencing. Anal. Biochem. *266*:198-204.
3. **Keshvara, L.M., C.C. Isaacson, T.M. Yankee, R. Sarac, M.L. Harrison, and R.L. Geahlen.** 1998. Syk- and Lyn-dependent phosphorylation of Syk on multiple tyrosines following B cell activation includes a site that negatively regulates signaling. J. Immunol. *161*:5276-5283.
4. **Meisenhelder, J., T. Hunter, and P. van der Geer.** 1999. Phosphopeptide mapping and identification of phosphorylation sites. *In* Current Protocols In Molecular Biology, Volume 4, Section 18.9. John Wiley & Sons, New York.
5. **Meshi, T., I. Moda, M. Minami, M. Okanami, and M. Iwabuchi.** 1998. Conserved ser residues in the basic region of the bZIP-type transcription factor HBP-1a(17): importance in DNA binding and possible targets for phosphorylation. Plant Mol. Biol. *36*:125-136.
6. **Rowan, B.G., N.L. Weigle, and B.W. O'Malley.** 2000. Phosphorylation of steroid receptor coactivator-1. J. Biol. Chem. *275*:4475-4483.
7. **Tagawa, T., T. Kuroki, P.K. Vogt, and K. Chida.** 1995. The cell cycle-dependent nuclear import of v-Jun is regulated by phosphorylation of a serine adjacent to the nuclear localization signal. J. Cell Biol. *130*:255-263.
8. **West, M.H. and W.M. Bonner.** 1984. Polyacrylamide gel electrophoresis of small peptides. Electrophoresis *5*:133-138.

9.**Xanthoudakis, S., G. Maio, F. Wang, Y.C. Pan, and T. Curran.** 1992. Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. EMBO J. *11*:3323-3335.

The authors thank Christopher T. Jones and Robert L. Geahlen for helpful discussions. This work was supported by PHS CA-78264 (E.J.T) and PHS T32 CA-09643 (C.D.D.). *Address correspondence to Dr. Elizabeth J. Taparowsky, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA. e-mail: ejt@bilbo.bio.purdue.edu*

Received 27 August 2002; accepted 1 October 2002.

Christopher D. Deppmann and Elizabeth J. Taparowsky
Purdue University
West Lafayette, IN, USA

Plastic Contaminant Masquerades as DNA in Mutation Detection by Denaturing HPLC

BioTechniques 34:59-60 (January 2003)

Denaturing high-performance liquid chromatography (DHPLC) has quickly become popular as a tool for the automated discovery of single base substitutions as well as small insertions and deletions (1,2). Under partially denaturing conditions, heteroduplices are retained for a shorter time than their corresponding homoduplices on a DNA separation matrix. We have identified a contaminant emanating from polypropylene PCR tubes that masquerades as DNA in DHPLC assays. While small amounts of plasticizer contaminants are well known anecdotally in DHPLC analyses, the contaminant detected here had a peak area 20–50 times that of the level of PCR products required for analysis.

The tube contaminant was identified when using 8 Tube Strips from Axygen Scientific (Union City, CA, USA) while amplifying for 40 cycles a 475-bp fragment of the Paraoxonase gene, PON-1. DHPLC was conducted on a Varian Helix System fitted with a 75-mm Helix Analysis Column packed with C18 alkylated silica (Varian, Walnut Creek, CA, USA). Standard operating procedures were used, utilizing 100 mM triethyammonium acetate (TEAA), pH 7.0, 0.1 mM EDTA as buffer A and 100 mM TEAA, pH 7.0, 0.1 mM EDTA, 25% (v/v) acetonitrile as buffer B (both from Varian), with a gradient of 50%–68% buffer B over 5.5 min. A similar contaminating peak has been observed using Axygen Scientific 96-Well PCR Plates (personal communication, Me-