SUMMARY

(In accordance with 40 CFR part 152, this summary is available
for public release after registration)

STUDY TITLE

Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from
Transgenic Soybean Event DAS-68416-4

AMENDED REPORT

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

B. W. Schafer, S. K. Embrey

REPORT COMPLETED ON

01-Sep-2009

Amended Report Date: September 29, 2010

PERFORMING LABORATORY

Regulatory Sciences and Government Affairs—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

081132

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To perform various toxicology, eco-toxicology, and biochemical characterization studies, large quantities of the PAT protein are required. Because it is technically infeasible to extract and purify sufficient amounts of PAT protein from transgenic plants, the protein was produced in a *Pseudomonas fluorescens* expression system. The purpose of this study was to characterize the recombinant PAT protein derived from both *P. fluorescens* and transgenic soybean plants.

Biochemical analyses were performed to characterize the PAT protein derived from microbial and transgenic plant test materials. The analyses performed were sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, and lateral flow strip testing. SDS-PAGE confirmed that the microbe- and plant-derived PAT proteins were at the expected molecular weight. Western blotting (and lateral flow test strips) confirmed that both test materials contained protein immunoreactive to antibodies specific to the PAT protein at the expected molecular weight. Non-transgenic soybean did not contain immunoreactive proteins.

Together, these biochemical tests indicate that the plant- and microbe-derived proteins are substantially equivalent, and therefore the microbe-derived protein is acceptable for use in regulatory studies.

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Not Applicable

AUTHOR(S)

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S. K. Embrey

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Total number of pages is 28 including 4.1R1 of 27

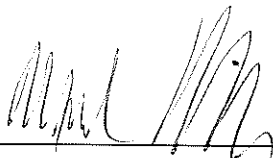## STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: PAT

Title: Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-68416-4

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A)(B), or (C).*

Company: Dow AgroSciences LLC

Company Agent: M. S. Krieger

Title: Regulatory Manager

Signature:

Date: 28 Sep 2010

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THESE DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

This report has been amended. This page was signed prior to or on the study completion date, but has been re-signed due to the modification.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-68416-4

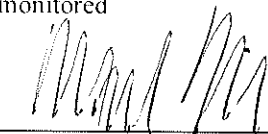Study Initiation Date: 04-Feb-2009   Study Completion Date   01-Sep-2009

*Amended Report Date: September 29, 2010

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
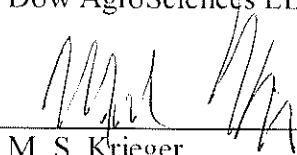FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160 with the following exceptions. The GLP status of the commercially available protein molecular markers and bovine serum albumin was unknown. The chain of custody of the standards was not monitored
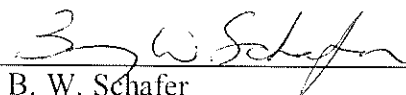
_____   28 Sep 2010
M. S. Krieger   Date
Sponsor
Dow AgroSciences LLC

_____   28 Sep 2010
M. S. Krieger   Date
Submitter
Dow AgroSciences LLC

_____   29 Sep 2010
B. W. Schafer   Report Completion Date
Study Director/Author
Dow AgroSciences LLC

*This report has been amended. The original report was completed and signed on 01-Sep-2009. The study director's signature and date reflect the date of the report amendment.

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Study ID: 081132

Title: Characterization of the Phosphinothricin Acetyltrasferase (PAT) Protein Derived from Transgenic Soybean Event DAS-68416-4

Study Initiation Date: 4-Feb-2009   **Study Completion Date:** 1-Sept-2009

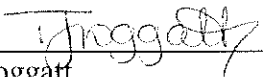   ***Amended Study**   29-Sept-2010

                                                Completion Date:

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
4-Feb-2009	4-Feb-2009	Protocol Review
4-Mar-2009	4-Mar-2009	Western Blot Analysis
21 & 24-Aug-2009	25-Aug-2009	Report and Raw Data Review; Test Substance Container and Sample Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

_____   29-Sept-2010 _____
Tracey Froggatt   Date
Dow AgroSciences, Quality Assurance

*The original report was completed and signed on 1-Sept-2009. The new signature and date reflects the date of the report amendment.

Summary of Amendment

Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-68416-4

Summary of amendment changes:

Summary Page: Page 1R1 of 3, the phrases AMENDED REPORT and Amended Report date were added.

Title page: Page 1R1 of 27, the phrases AMENDED REPORT, Amended Report date.

Page 3R1 of 27: The report amended date and a statement describing the original signature dates were added.

Page 4R1 of 27: A new QA page was added

Page 4.1R1 of 27: This page is added to describe the changes.

Page 5R1 of 27: New signatures were collected.

Page 10R1 of 27: The seed generation was changed from T4 to T5.
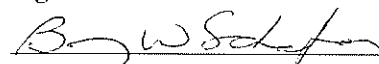
Reason for Amendment:
It was discovered that the generation number of the seeds was not correct due to an error in Variety database used to determine the generation number of AAD-12 soybean (event DAS-68416-4) seeds. The correct generation number will be documented in the amended report and study files.

Impact on Study:
This amendment has a positive impact on the study as the correct generation number will be documented in the amended report and study files. The correction has no impact on the study because the nature of the seeds has not changed.
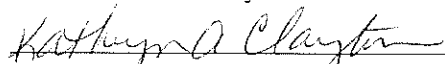
Signatures:

B. W. Schafer, Study Director
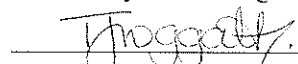
Date: 29. SEP. 2010

K. A. Clayton, Manager
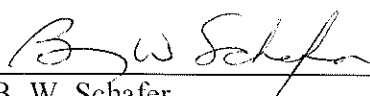
Date: 28 Sept 2010

T. Froggatt, Quality Assurance
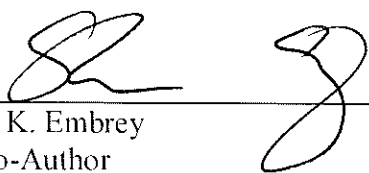
Date: 29-Sept-2010

SIGNATURE PAGE

B. W. Schafer
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Dow AgroSciences LLC

29. Sep 2010
Date

S. K. Embrey
Co-Author
Dow AgroSciences LLC
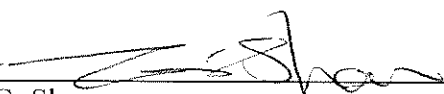
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R. A. Herman
Peer-Reviewer
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29-Sept 2010
Date

G. Shan
Science Leader
Dow AgroSciences LLC

28 Sept 2010
Date

K. A. Clayton
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28 Sept 2010
Date

STUDY PERSONNEL

Title: Characterization of the Phosphinothricin Acetyltransferase (PAT) Protein Derived from Transgenic Soybean Event DAS-68416-4

Study Director: Barry W. Schafer

Analyst: Shawna K. Embrey

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ABSTRACT

Soybean plants have been modified by the insertion of an herbicide-resistant selectable marker gene *pat*, isolated from the bacterium *Streptomyces viridochromogenes*, which expresses the enzyme phosphinothricin acetyltransferase or PAT. The expressed PAT protein acetylates phosphinothricin, or its precursor demethylphosphinothricin, conferring tolerance to a chemically synthesized phosphinothricin such as the herbicide glufosinate-ammonium. Acetylation converts phosphinothricin to an inactive form that is no longer toxic to soybean plants. Individual soybean plants tolerant to glufosinate-ammonium herbicide can be identified in the field through application of the herbicide to leaves.

To perform various toxicology, eco-toxicology, and biochemical characterization studies, large quantities of the PAT protein are required. Because it is technically infeasible to extract and purify sufficient amounts of PAT protein from transgenic plants, the pr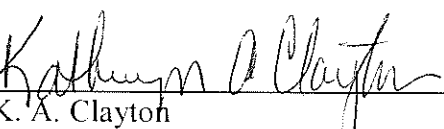otein was produced in a *Pseudomonas fluorescens* expression system. The purpose of this study was to characterize the recombinant PAT protein derived from both *P. fluorescens* and transgenic soybean plants.

Biochemical analyses were performed to characterize the PAT protein derived from microbial and transgenic plant test materials. The analyses performed were sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, and lateral flow strip testing. SDS-PAGE confirmed that the microbe- and plant-derived PAT proteins were at the expected molecular weight. Western blotting (and lateral flow test strips) confirmed that both test materials contained protein immunoreactive to antibodies specific to the PAT protein at the expected molecular weight. Non-transgenic soybean did not contain immunoreactive proteins.

Together, these biochemical tests indicate that the plant- and microbe-derived proteins are substantially equivalent, and therefore the microbe-derived protein is acceptable for use in regulatory studies.

INTRODUCTION

Soybean plants have been modified by the insertion of an herbicide-resistant selectable marker gene *pat*, isolated from the bacterium *Streptomyces viridochromogenes*, which expresses the enzyme phosphinothricin acetyltransferase or PAT. The expressed PAT protein acetylates phosphinothricin, or its precursor demethylphosphinothricin, conferring tolerance to a chemically synthesized phosphinothricin such as the herbicide glufosinate-ammonium. Acetylation converts phosphinothricin to an inactive form that is no longer toxic to soybean plants. Individual soybean plants tolerant to glufosinate-ammonium herbicide can be identified in the field through application of the herbicide to leaves.

To perform various toxicology, eco-toxicology, and biochemical characterization studies, large quantities of the PAT protein are required. Because it is technically infeasible to extract and purify sufficient amounts of PAT protein from transgenic plants, the protein was produced in a *Pseudomonas fluorescens* expression system. The purpose of this study was to characterize the recombinant PAT protein derived from both *P. fluorescens* and transgenic soybean plants.

The biochemical and immunological methods employed in this study are among those that have been well established for protein analysis. SDS-PAGE separates proteins based on the apparent molecular weight (mass). Western blotting of proteins to a nitrocellulose membrane following SDS-PAGE, and immunodetection with a protein specific antibody is widely used to identify the authenticity of a molecule in a crude preparation (Burnette, 1981). Commercially available lateral flow test strips provide additional evidence of the authenticity of the PAT protein by reacting immunochemically with antibodies that are sensitive and specific for the PAT protein.

MATERAILS AND METHODS

Test Substance/Test System:

The test substance was the PAT protein expressed and extracted from tissues grown from the T5 seeds of transgenic soybean event DAS-68416-4 (Source ID: YX08CX010827.001). The seeds were planted on 16-Sep-2008 and harvested on 19-Nov-2008. After harvest, the tissues were frozen and lyophilized for short term storage at -80 °C. The presence of PAT protein in the soybean tissue was confirmed by a commercially available Lateral Flow Membrane Strip Assay kit (Product #: AS 014 LSS) from EnviroLogix Inc. (Portland, ME).

Control Substances:

1.   The control substance used in this study was a non-transgenic soybean plant extract (*Glycine max* cv Maverick). Seeds of the Maverick soybean line (Source ID: YX07KX002114) were planted, grown, harvested and processed under the same conditions as the transgenic plants described above. The absence of PAT protein in the soybean tissues was confirmed by a commercially available Lateral Flow Membrane Strip Assay kit from EnviroLogix Inc.

2.   Recombinant PAT microbial protein, (Lot #: 060519), molecular weight: 20.5 kDa. The sample contained 300 µg/mL of PAT protein (Schafer, 2006). The microbial preparation was produced and purified from recombinant *Pseudomonas fluorescens* strain DC454 at the Dow AgroSciences Core Biotech R&D facility in Indianapolis, IN (Snodderley, 2006). An aliquot of the purified sample was sent to the Test Substance Coordinator at Dow AgroSciences located in Indianapolis and the material was designated TSN105742.

Reference Substances

The commercially available reference substances used in this study are listed in the following table:

Reference Substance	Product Name	Lot Number	Assay	Reference
Bovine Serum Albumin Fraction V (BSA)	Pre-diluted BSA protein assay standard set	FH71884E	SDS-PAGE	Pierce Cat #: 23208
Molecular Weight Markers	Mark12 unstained protein standards	470020	SDS-PAGE	Invitrogen Cat #: LC5677, Molecular Weight Markers of 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31.0, 21.5, 14.4, 6.0, 3.5 and 2.5 kDa
Prestained Molecular Weight Markers	Novex Sharp prestained protein standards	469212	SDS-PAGE and Western Blot	Invitrogen Cat #: LC5800, Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa

Lateral Flow Test Strip Assay

The soybean leaf tissues of the transgenic and nontransgenic events were harvested fresh on November 19, 2008 and were frozen, lyophilized and stored at approximately –80 °C until use. To confirm the presence/absence of the PAT protein in the pooled tissues, approximately 30 mg of the lyophilized tissues (Event DAS-68416-4 and Maverick) were weighed into 1.5-mL microfuge tubes and tested by the lateral flow test strip assay as described by EnviroLogix (Portland, ME). Briefly, the soluble proteins were extracted by adding 0.25 mL of extraction buffer and grinding with a disposable pestle. The tubes were capped and further mixed by shaking for ~10 seconds. The resulting supernatants were clarified by centrifuging the samples for 5 minutes at 20,000xg. The test strips were then incubated in the clarified extracts for 5 minutes to develop. After the assay was complete, the strips were removed and allowed to air dry and the results were recorded.

SDS-PAGE and Western Blot

SDS-PAGE analysis of the transgenic and nontransgenic Maverick soybean extracts was performed with Bio-Rad Criterion gels (Cat #: 345-0123) fitted in a Criterion Cell gel module (Bio-Rad Cat #: 165-6001) with MES running buffer (Bio-Rad Cat #: 161-0789). Extracts were prepared in a bead mill (Geno-Grinding, Spex, Model #: 2000) with ~70 mg/mL of tissue and steel ball bearings in a PBST based buffer (Table 1) for 3 minutes in a chilled Teflon microfuge tube holder. The supernatants were clarified by centrifuging for 5 minutes at 20,000×g, and then 120 µL of each extract was mixed with 30 µL of 5x Laemmli sample buffer [LSB, 2% SDS, 50 mM Tris pH 6.8, 0.2 mg/mL bromophenol blue, 50% (w/w) glycerol containing 10% freshly added 2-mercaptoethanol (Bio-Rad, Cat #: 161-0710)]. Samples were heated for 5 minutes at ~95 °C, and after a brief centrifugation, 40 µL of the supernatant was loaded directly on the gel. The reference standard, microbe-derived PAT (TSN105742), and control standard, BSA (Pierce Cat #: 23208), were diluted with Bio-Rad 2x LSB (Bio-Rad Cat #: 161-0737 containing 5% 2-mercaptoethanol) and processed as described earlier. The electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes using MES running buffer. After separation, the gel was cut in half and one half was stained with Thermo Scientific GelCode Blue protein stain (Cat #: 24592) and scanned with a densitometer (Molecular Dynamics, Personal Densitometer Si) to obtain a permanent record of the image. The remaining half of the gel was electro-blotted to a nitrocellulose membrane (Bio-Rad, Cat #:162-0233) with a Criterion trans-blot electrophoretic transfer cell (Bio-Rad Cat#: 170-4070) for ~60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad (Cat #: 161-0734). After transfer, the membrane was cut in half and one half was probed with a PAT specific polyclonal rabbit antibody (EnviroLogix Lot #: 69:74A, 1.0 mg/mL) and the remaining half was probed with a PAT specific monoclonal antibody (DAS Lot #155AD4, 1.2 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase (Pierce, Cat #: 31460), and goat anti-mouse IgG (H+L) and horseradish peroxidase (Bio-Rad, Cat#: 170-6516) were used as the secondary antibodies, respectively. GE Healthcare chemiluminescent substrate (Cat #:

RPN2132) was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to Thermo Scientific (Cat #: 34091) CL-XPosure detection film for various time points and subsequently developed with an All-Pro 100 Plus film developer (Melville, NY).

Statistical Treatment of Data

No statistical analyses were conducted during this study.

RESULTS AND DISCUSSION

Lateral Flow Test Strip Assay

The presence of the PAT protein in the pooled leaf tissue (T4) of DAS-68416-4 was confirmed using commercially prepared lateral flow test strips from EnviroLogix. The strips easily discriminated between transgenic and nontransgenic plants as the non-transgenic extracts of Maverick did not contain detectable amounts immunoreactive protein. This result was also confirmed by western blot analysis.

SDS-PAGE and Western Blot Analysis

In the toxicology-lot preparation of *P. fluorescens*-produced PAT protein (TSN105742), the major protein band, as visualized on Coomassie stained SDS-PAGE gels, was approximately 20.5 kDa (Figure 1 Panel A). As expected, the corresponding soybean-derived PAT protein was visualized by immunospecific polyclonal and monoclonal antibodies at an identical size to the

microbe-expressed proteins (Figure 1, Panel B and C). The microbe-derived PAT protein also showed a positive signal of the expected size by polyclonal and monoclonal antibody western blot analysis (Figure 1, Panel B and C). In the PAT western blot analysis, no immunoreactive proteins were observed in the control Maverick extract and no alternate size proteins (aggregates or degradation products) were seen in the transgenic samples (Figure 1, Panel B and C). The monoclonal antibody did detect a small amount of the PAT dimer in the microbe-derived protein. These results add to the evidence that the protein expressed in soybean is not post-translationally modified which would have added to the overall protein molecular weight.

CONCLUSIONS

The results of this study demonstrated that both the transgenic soybean-plant extract and the microbe-derived PAT toxicological lot contained the intact, full-length PAT protein. This was confirmed by SDS-PAGE molecular-weight approximation, western blot analysis and commercially available lateral flow strip tests. Together, these biochemical tests indicate that the plant- and microbe-derived proteins are substantially equivalent, and therefore the microbe-derived protein is acceptable for use in regulatory studies

REFERENCES

Burnette, W. N. 1981. Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195-203.

Schafer, B. W. (2006) Certificate of Analysis for Test/Reference/Control Substance TSN105742. Unpublished report of Dow AgroSciences, Study #: BIOT063302.

Snodderley, E. (2006) Expression and Purification of Recombinant Phosphinothricin *N*-Acetyl Transferase (PAT), TSN 105742, from *Pseudomonas fluorescens* DC454. Unpublished report of Dow AgroSciences, Study #: 238452.

Table 1.   Soybean-Derived PAT Extraction Buffer Composition

Amount	Ingredient	Catalog Numbers
3.0 mL	Phosphate Buffered Saline with 0.05% Tween[a] 20, pH 7.4	Sigma Cat #: P3563
30 µL	0.5 M EDTA	AccuGENE Cat #: 51234
50 µL	Plant protease cocktail inhibitor	Sigma Cat #: P9599
5.0 µL	β-mercaptoethanol	Bio-Rad Cat#: 161-0710

Note: the extraction buffer was prepared immediately before use.

Panel A: SDS-PAGE   **Panel B:** Polyclonal antibody western blot   **Panel C:** Monoclonal antibody western blot

Lane	Sample	Amount
M	Invitrogen Mark12 molecular weight markers	10 μL
1	Nontransgenic (Maverick) soybean extract	40 μL
2	Transgenic (Event DAS-68416-4) soybean extract	40 μL
3	Microbe-derived PAT protein (TSN105742)	750 ng gel, 35 ng blot
4	Bovine serum albumin (BSA)	780 ng gel
P	Novex Sharp prestained molecular weight markers	10 μL

SDS-PAGE analysis of the transgenic and nontransgenic soybean extracts was performed with Bio-Rad Criterion gels (Cat #: 345-0123) fitted in a Criterion Cell gel module (Cat #: 165-6001) with MES running buffer (Bio-Rad Cat #: 161-0789). Extracts were prepared by Geno-Grinding (Spex, Model #: 2000) ~70 mg/mL of tissue with steel ball bearings in a PBST based buffer for 3 minutes in a chilled Teflon microfuge tube holder. The supernatants were clarified by centrifuging the samples for 5 minutes at 20,000×g and 120 μL of each extract was mixed with 30 μL of 5x Laemmli sample buffer containing 10% freshly added 2-mercaptoethanol and heated for 5 minutes at ~95 °C. After a brief centrifugation, 40 µL of the supernatant was loaded directly on the gel. The reference standard, microbe-derived PAT (TSN105742), and control standard, BSA, were diluted with 2x Laemmli sample buffer containing 5% 2-mercaptoethanol and processed as described earlier. The electrophoresis was conducted at a constant voltage of 150 V for ~60 minutes. After separation, the gel was cut in half and one half was stained with Pierce GelCode Blue protein stain (Cat #: 24592) and scanned with a densitometer (Molecular Dynamics, Personal Densitometer Si) to obtain a permanent record of the image. The remaining half off the gel was electro-blotted to a nitrocellulose membrane (Bio-Rad, Cat #:162-0233) with a Criterion trans-blot electrophoretic transfer cell for 60 minutes under a constant voltage of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. After transfer, the membrane was cut in half and one half was probed with a PAT specific polyclonal rabbit antibody (EnviroLogix Lot #: 69:74A, 1.0 mg/mL) and the remaining half was probed with a PAT specific monoclonal antibody (DAS Lot #155AD4, 1.2 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase (Pierce Chemical, Cat #: 31460) and goat anti-mouse IgG (H+L) and horseradish peroxidase (Bio-Rad, Cat#: 170-6516) were used as the secondary antibodies respectively. GE Healthcare chemiluminescent substrate (Cat #: RPN2132) was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to Thermo Scientific CL-XPosure detection film (Cat #: 34091) for various time points and subsequently developed with an All-Pro 100 Plus film developer.

Figure 1.   SDS-PAGE and western blots of transgenic soybean Event DAS-68416-4 and nontransgenic Maverick.

APPENDIX

"Western Blotting": Electrophoretic Transfer of Proteins from Sodium Dodecyl Sulfate–Polyacrylamide Gels to Unmodified Nitrocellulose and Radiographic Detection with Antibody and Radioiodinated Protein A

W. Neal Burnette[1]

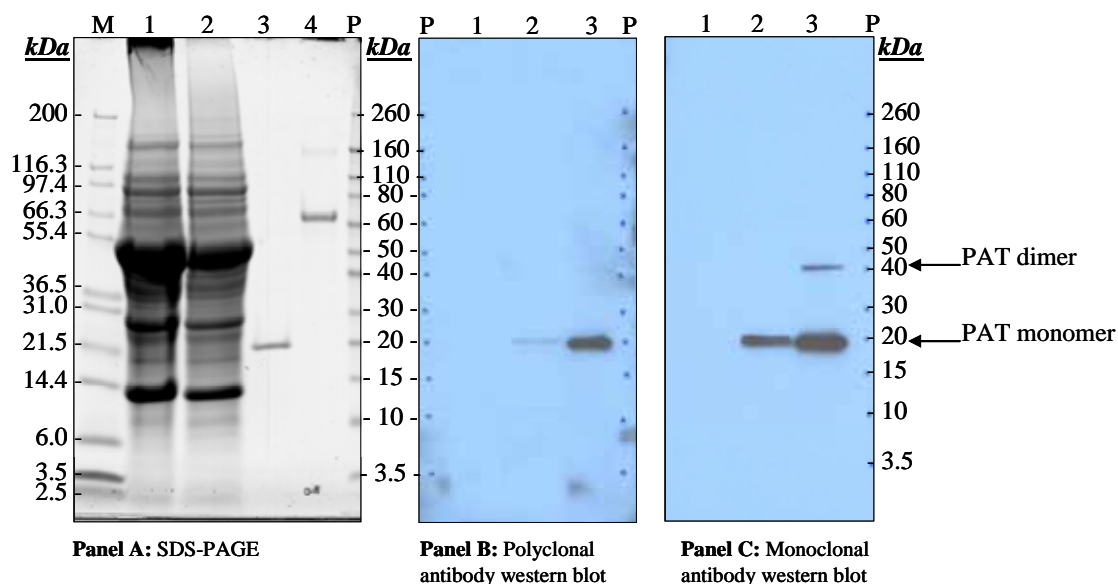*Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104*

A simple and efficient procedure was employed for the electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to sheets of pure, unmodified nitrocellulose. Immobilized proteins could then be radiographically visualized *in situ* by reaction with specific antibody and the subsequent binding of radioiodinated *Staphylococcus* protein A to the immune complexes. The detection of murine leukemia virus antigens in complex cellular lysates was used to demonstrate the efficacy of this technique.

A powerful tool in molecular genetics has been the blotting technique of Southern (1) in which electrophoretically fractionated DNA can be immobilized onto nitrocellulose filters and used to examine complementary sequences by hybridization *in situ*. An adaptation of the "Southern" blot is the covalent attachment of fractionated RNA (or DNA) to diazobenzyloxymethyl paper (DBM paper)[2] in order to probe for complementary DNA sequences ("Northern" blotting) (2). Although various proteins have been detected after fractionation on polyacrylamide gels using enzyme substrates (3) or specific antibody (4), only recently have attempts been made to immobilize gel-fractionated proteins on a solid phase (5–7). These techniques involve either passive diffusion or the preparation and activation of DBM paper to achieve covalent immobilization.

Our laboratory has been interested in finding a simple yet reproducible transfer technique for use in the immunological detection and characterization of the products encoded by murine leukemia virus (MuLV) genomes. In this regard, Towbin *et al.* (8) recently described an elegant and straightforward method for the electrophoretic transfer of ribosomal proteins from polyacrylamide–urea gels to sheets of unmodified nitrocellulose and the radioautographic detection of specific antigens on such gel replicas with radiolabeled antibodies. Most types of biochemical and immunochemical analyses, however, make use of sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis to identify and characterize proteins by their relative molecular weights. As described below, it was found that the rate of electrophoretic elution of proteins from SDS-containing gels was itself molecular weight dependent. By making certain adaptations to the transfer method of Towbin *et al.* (8), it was possible

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[1] Present address: Tumor Virology Laboratory, The Salk Institute, Post Office Box 85800, San Diego, Calif. 92138.

[2] Abbreviations used: DBM paper, diazobenzyloxymethyl paper; MuLV, murine leukemia virus; SDS, sodium dodecyl sulfate; IPA, radioiodinated *Staphylococcus* protein A; NP-40, Nonidet P-40; 2-DGE, two-dimensional gel electrophoresis; BSA, bovine serum albumin; IgG, immunoglobulin G.

to achieve essentially complete and quantitative elution of most proteins from SDS–gels to nitrocellulose with no loss in electrophoretic resolution. The use of radioiodinated *Staphylococcus* protein A (IPA), instead of radiolabeled primary or secondary antibodies, greatly simplified and enhanced the radioautographic detection of immune complexes on blots reacted with specific immune sera.

In addition to the general technique of electrophoretic transfer and immunological detection, various applications of the method are discussed. Certain problems are also identified, particularly those regarding the reuse of the gel blots, replica distortion, and antigen denaturation. With due respect to Southern (1), the established tradition of "geographic" naming of transfer techniques ("Southern," "Northern") is continued; the method described in this manuscript is referred to as "Western" blotting.

MATERIALS

AKR MuLV-infected C57BL/6 E♂G2 cells and Moloney MuLV-infected C3H cells were donated by R. Nowinski of this Center. ¹⁴C-Labeled molecular-weight-marker proteins and sodium [¹²⁵I]iodide were purchased from New England Nuclear (Boston, Mass.). *Staphylococcus* protein A from Pharmacia (Piscataway, N. J.) was radioiodinated as described by Hunter (9). Generally, IPA can be stored in aliquots for up to 2 weeks at −70°C in solutions containing 5% bovine serum albumin without significant loss in reactivity toward immunoglobulin. Acrylamide and bisacrylamide were from Polysciences (Warrington, Pa.) and Sequanal-grade SDS was from Pierce Chemical Company (Rockford, Ill.). Grade 470 filter paper and sheets of nitrocellulose of various pore sizes were purchased from Schleicher and Schuell (S&S, Keene, N. H.). The Canalco gel destaining apparatus is sold by Miles Laboratories

(Elkhart, Ind.); only the electrophoresis chamber and plate electrodes (sold separately) are necessary for the transfer technique. Gel electrophoretic apparatuses were manufactured to specifications by Biocraft (Peekskill, N. Y.). Rabbit anti-p30 sera were generously provided by H. Fan of the Salk Institute and by J. Ihle of the Frederick Cancer Research Center.

METHODS

Sample preparation. AKR MuLV-infected C57BL/6 E♂G2 cells and Moloney MuLV-infected C3H cells were washed three times in phosphate-buffered saline. Cell pellets, collected by centrifugation, were suspended at a density of no greater than 20–30 × 10⁶ cells/ml in a lysis buffer (10) containing 0.5 M urea, 2% Nonidet P-40 (NP-40; Shell Chemical Co., West Orange, N. J.), 2% Ampholine pH 3.5–10 (LKB Instruments, Stockholm) and 5% 2-mercaptoethanol. The cells were frozen and thawed three times and insoluble debris removed by centrifugation for 10 min in an Eppendorf centrifuge. Lysates were stored at −70°C until used. These samples could be applied directly to isoelectric focusing gels for two-dimensional gel electrophoresis (2-DGE). For SDS gel electrophoresis, small sample volumes could be diluted directly into SDS–gel sample buffer (11) or, for larger sample volumes, could be precipitated with cold trichloroacetic acid, the precipitates collected by centrifugation, and washed in cold acetone before resuspension in sample buffer.

Gel electrophoresis. SDS gel electrophoresis was performed in 10, 12.5, or 5–20% linear gradient acrylamide slab gels as previously described (11,12). 2-DGE is a cathode-directed isotachophoretic step coupled with SDS gel electrophoresis which has been used in this laboratory for the analysis of monoclonal immunoglobulins (13).

Electrophoretic transfer. The transfer procedure has been adapted from Towbin

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et al. (8) for the quantitative recovery of proteins from SDS-containing polyacryl-amide gels. Briefly, a "sandwich" is pre-pared with the following successive layers: (i) a porous polyethylene sheet (Bel-Art), 13.5 × 14 cm and 1.6 mm thick; (ii) three thicknesses of filter paper (S&S grade 470), 13.5 × 10 cm; (iii) the SDS–polyacryl-amide slab gel (13.5 × 9.5 cm with the stacking gel removed); (iv) a nitrocellulose sheet (S&S BA83, 0.2 μm) cut to the size of the gel; (v) three more thicknesses of filter paper; and finally (vi) another sheet of po-rous polyethylene. All components in con-tact with the slab gel are prewetted in the electrode solution composed of 20 mM Tris base, 150 mM glycine, and 20% methanol (8). This solution should be degassed briefly under vacuum before use. The sand-wich is secured with thick rubber bands and inserted between the electrodes of the Can-alco gel destainer with the nitrocellulose toward the anode. The chamber is filled with electrode solution and electrophoretic transfer accomplished at 6–8 V/cm (with respect to electrode separation) for 16–22 h using a Buchler 3-1155 power supply. Re-circulation of the electrode solution is not necessary.

Staining, radioautography, and fluorog-raphy. For direct visualization of unlabeled proteins, the nitrocellulose sheet may be stained for 5 min in a solution containing 0.2% Coomassie brilliant blue R-250, 40% methanol, and 10% acetic acid. Rapid de-staining is accomplished in 90% methanol and 2% acetic acid as described (8,14). Care must be exercised during destaining be-cause the nitrocellulose will disintegrate if left in the acidic methanol for much longer than 5 min. The destained sheet is rinsed in water and blotted dry for 1–2 h between sheets of S&S grade 470 paper. Hot air dry-ing should be avoided. Protein preparations radiolabeled with [^{35}S]methionine, ^{125}I, or to high specific activities with ^{14}C-labeled precursors may be directly visualized by ra-dioautography after drying the nitrocellu-

lose replica. ^{14}C-Labeled and ^{3}H-labeled proteins may be visualized by fluorography utilizing the fluorographic cocktail "En-hance" (New England Nuclear), followed by intensifying screen-enhanced radioau-tography (15).

Binding of antibody and IPA to nitrocel-lulose-immobilized proteins. Immediately following transfer, the nitrocellulose sheet is immersed in a solution containing 0.9% NaCl, 10 mM Tris–HCl, pH 7.4 (Tris–sa-line), and 5% fraction V bovine serum albu-min (BSA), and incubated at 40°C for 30 min on a rocking platform. The sheet is transferred to a fresh solution of Tris–sa-line with BSA containing the appropriate antiserum and incubated for 90 min at room temperature on a rocking platform. The ni-trocellulose is then washed with rocking for 10 min in 200 ml of Tris–saline without BSA, for 20 min in two changes of 200 ml each of Tris–saline containing 0.05% NP-40, and again for 10 min in 200 ml of Tris–saline alone. The sheet is immersed in fresh Tris–saline with 5% BSA containing 2–5 × 10^5 cpm of IPA/ml. Binding of IPA is allowed to occur for 30 min with rocking at room temperature. The radioactive solution is aspirated and the nitrocellulose sheet again rinsed as described above. The sheet is briefly blotted with paper towels, wrapped in Glad-Wrap, and exposed at −70°C to Kodak XR film utilizing a GAFMED Rarex Mid Speed intensifying screen.

RESULTS AND DISCUSSION

Efficiency of Electrophoretic Transfer and Immobilization of Proteins

Figure 1 shows the indirect radioauto-graph of ^{14}C-labeled proteins on unmodified nitrocellulose following their transfer at 8 V/cm for 22 h from a 12.5% polyacrylamide –SDS gel. Compared to the electrophoretic transfer of proteins to DBM paper, Western blotting with unmodified nitrocellulose gives markedly better resolution, is signifi-
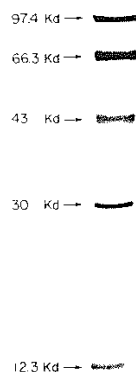
97.4 Kd →

66.3 Kd →

43 Kd →

30 Kd →

12.3 Kd →

FIG. 1. Western blot of ^{14}C-labeled proteins. Approximately 6 nCi each of ^{14}C-labeled phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and cytochrome *c* were fractionated in a 12.5% polyacrylamide SDS–gel. Following electrophoresis, the proteins were transferred to 0.20 μM nitrocellulose paper as described in the text and the blot exposed to Kodak XR film.

cantly easier to perform, and does not require preequilibration of the gel nor diazotization of the nitrocellulose (R. Eisenman and W. Mason, personal communication). Six different grades of S&S nitrocellulose paper, with pore sizes ranging from 0.025 to 0.45 μm, were examined for efficiency of protein immobilization. No significant difference was found among them in resolution or in effective adsorption of marker proteins phosphorylase *b* (97.4 kdalton) bovine serum albumin (66.3 kdalton), ovalbumin (43 kdalton), and carbonic anhydrase (30 kdalton). However, the adsorption of cytochrome *c* (12.5 kdalton) to 0.45 μm nitrocellose was consistently 20–30% less than to other nitrocelluloses. Consequently, nitrocellulose with a pore size of 0.2 μm (S&S BA83) was employed for all subsequent studies. This nitrocellulose paper exhibited a protein adsorption capacity of about 2 μm/mm², far exceeding the

amount necessary for detection by even less-sensitive staining methods (e.g., Coomassie brilliant blue, amido black).

In preliminary experiments it was apparent that an electrophoretic blotting time of 1 h at 6–8 V/cm, as suggested by Towbin *et al.* (8) and Bittner *et al.* (7), was not sufficient for complete transport of most proteins out of SDS-containing polyacrylamide gels. The rate of transfer is, in fact, dependent upon the apparent molecular weight of individual proteins, i.e., lower-molecular-weight polypeptides leave the gel and are deposited on the nitrocellulose faster than higher-molecular-weight proteins at a given accelerating voltage. Figure 2 qualitatively illustrates the necessity of longer blotting times. The upper panels of Fig. 2 show the residual radioactivity remaining in 10% polyacrylamide–SDS gels after transfer at 8 V/cm for 0, 1, 4, 12, and 22 h. The lower panels are the corresponding nitrocellulose blots. Quantitative transfer as a function of molecular weight can be demonstrated by cutting the appropriate radiolabeled bands
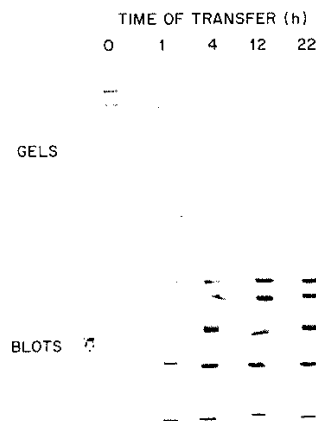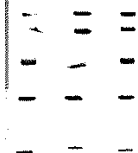
TIME OF TRANSFER (h)

0   1   4   12   22

GELS

BLOTS

FIG. 2. Transfer of proteins as a function of electrophoresis time. ^{14}C-Labeled marker proteins were fractionated by 10% SDS gel electrophoresis and electrophoretically transferred to nitrocellulose at 8 V/cm for the times indicated. The upper panels are radioautograms showing the residual radioactivity remaining in the gels after transfer. The lower panels are the corresponding blots for each time point.

detection by even methods (e.g., Coomido black).

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from each of the blots and determining radioactivity by liquid scintillation counting. Figure 3 illustrates the kinetics of transfer for two proteins, phosphorylase *b* (97.4 kdalton) and carbonic anhydrase (30 kdalton). It can be seen that quantitative transfer of both proteins is only approached after 12 h. Mass dependence of transfer is not affected by presoaking the gel before blotting to remove residual detergent. Indeed, equilibration in a solution other than the electrode solution can result in swelling or shrinking of the gel during blotting with consequent distortion of the replica pattern (see below). For 1.5-mm-thick gels, therefore, 20–22 h of electrophoresis at 6–8 V/cm is sufficient for greater than 90% transfer of all proteins up to about 100,000 daltons.

Accelerating voltages higher than 10 V/cm are not recommended for electrophoretic transfer because of the attendant joule heating of the gel. Constant-current electrophoresis is also satisfactory if the voltage limit is not exceeded. Although there is no separation of the cathodic and anodic chambers in the Canalco destainer,
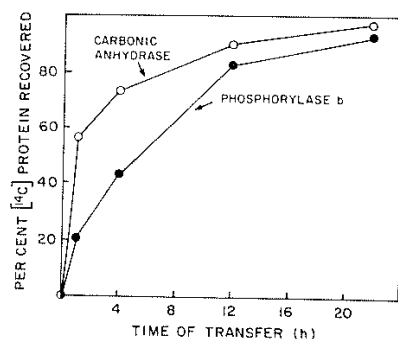

FIG. 3. Electrophoretic transfer time as a function of molecular weight. ^{14}C-Labeled phosphorylase *b* (97.4 kdalton) and carbonic anhydrase (30 kdalton) were fractionated by 10% SDS gel electrophoresis and subjected to electrophoretic blotting for 1, 4, 12, and 22 h at 8 V/cm. The individual protein bands were visualized by radioautography, cut from the nitrocellulose blots, and the radioactivity at each time point determined by liquid scintillation counting.
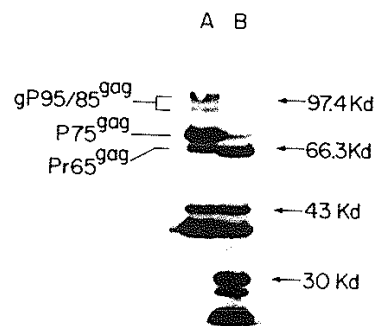

FIG. 4. Western blot and antibody–IPA detection of intracellular MuLV-specific antigens. Lysates of AKR MuLV-infected E♂G2 mouse cells and Moloney MuLV-infected C3H mouse cells were subjected to SDS gel electrophoresis in a 10% polyacrylamide slab gel and the fractionated proteins subsequently transferred to nitrocellulose paper. The blot was then reacted with rabbit antiserum directed against the major internal structural protein (p30) of MuLV and the immune complexes illuminated with IPA followd by radioautography. Lane A contained lysate equivalent to 3×10^5 E♂G2 cells and lane B to 1×10^5 C3H cells. Exposure time of this radioautogram was 15 min.

a pH gradient of 6.8–8.8 is set up across the plate electrodes during electrophoresis. However, this does not adversely affect transfer and recirculation of the electrode solution is not necessary. Transfer of proteins from isoelectric focusing slab gels (1.5 mm thick) containing urea can be accomplished in 4–6 h at the same voltage using an electrode solution of 0.7% acetic acid and reversing the electrodes.

Immunodetection of Blotted Proteins

Figure 4 is the Western blot and antibody–IPA illumination of MuLV-specific products found in AKR virus-infected E ♂ G2 mouse cells and Moloney MuLV-infected C3H mouse cells. The 65,000-dalton polyprotein precursor (Pr65^{gag}) of the major MuLV structural proteins (see Ref. (16)) is easily detected in both cell lines with a rabbit antiserum directed against a constituent of the polyprotein, the viral capsid polypeptide p30 (17,18). Here the intracellular viral proteins can be seen in a steady-state con-

dition not fully achievable by metabolic radiolabeling. The immunoreactive band identified as P75^{gag} in Fig. 4 is probably the glycopolyprotein precursor (gPr80^{gag}) to the glycosylated cell surface antigens gP95/85^{gag} (19,20). Although P75^{gag} is structurally and antigenically related to Pr65^{gag}, it is believed to be a separately initiated translation product of the viral *gag* gene (21). The lower-molecular-weight polypeptides in Fig. 4 are specific proteolytic cleavage products in the processing of Pr65^{gag} to the individual structural proteins of the virion (16).

Although radioiodinated antibodies (both primary and secondary) can be used to detect the immobilized antigens, IPA was chosen for the convenience and simplicity of preparing a single, standardizable radiolabeled reagent capable of detecting most types of immune complexes. The varying levels of radiographic "background" seen with different antisera preparations after reaction with IPA is due to nonspecific binding of immunoglobulin to nitrocellulose. The signal-to-noise ratio is therefore a function of the relative titers of specific immune antibodies and nonimmune IgG in each serum. Various protein solutions and protein admixtures have been used in efforts to "block" the nonspecific adsorption of immunoglobulin to the nitrocellulose and thereby increase the signal-to-noise ratio of the IPA-immune complexes. Although none of these mixtures (e.g., ovalbumin, avian globulins, gamma globulin-free bovine serum albumin) have duplicated the nitrocellulose binding of mammalian immunoglobulins, solutions of 5% bovine albumin (fraction V) have given satisfactory results and are the most convenient and inexpensive. Lengthening or increasing the number of washings after either antibody or IPA binding generally had no effect toward decreasing background. Since the Fc portion of rabbit IgG has a greater affinity for protein A than the immunoglobulins of most other animal spe-

cies (22), one can use much higher dilutions of rabbit antisera compared to the immune sera of other animals (e.g., goat); there is, however, no increase in the signal-to-noise ratio. Most antisera, diluted in Tris–saline–BSA and stored at $-20°C$, can be reused many times for reaction with blotted proteins. One such antiserum dilution was used more than 40 times over a 10-month period with no apparent diminution in specific immunoreactivity. Freshly radioiodinated protein A at $2–5 \times 10^5$ cpm/ml was routinely employed to illuminate the immune complexes since most results could be seen after 30–60 min of intensifying screen-enhanced radioautography. Smaller inputs of IPA require a consequent increase in radiographic exposure time. Neither the amount nor the specific activity of IPA had any significant effect on the signal-to-noise ratio.

Sensitivity of Immunodetection

It must be emphasized that sensitivity in Western blotting is primarily a function of the specific antibody titer of the immune serum being utilized. From studies of avian leukosis viruses (see Ref. (23)) it can be calculated that as little as 1–2 ng of a specific viral protein (p27) is detectable on blots with the appropriate hyperimmune rabbit serum (1:50 final dilution) exhibiting low nonspecific binding to nitrocellulose. This particular antiserum was capable of precipitating approximately 1 ng of ^{125}I-labeled avian myeloblastosis virus p27 at a final dilution of 1:20,000 in a standard 100-μl radioimmunoprecipitation assay (24). The rabbit antiserum used for the experiments shown in Fig. 4 had a radioimmunoprecipitation titer of about 1:12,000 for 0.8 ng of AKR MuLV p30. At a final dilution of 1:250 in Western blotting and a radiographic exposure time of 15 min after reaction with 2×10^5 cpm of IPA/ml, this hyperimmune serum detected the viral polyproteins in lysates equivalent to 3×10^5 E♂G2 cells

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(Fig. 4, lane A) and 1 × 10^5 C3H cells (lane B). Other experiments with this same anti-serum showed it easily capable of detecting Moloney MuLV antigens from as little as 10^3 C3H cells.

Other Applications of the Western Blot

Figure 5 is the Western blot of the 2-DGE of E♂G2 and C3H cell lysates. There is no loss of resolution in the focused spots after transfer and reaction with antibody–IPA. Western blotting used in conjunction with 2-DGE offers high sensitivity, two indepen-dent parameters of fractionation, mainte-nance of point-to-point resolution, simplic-ity of technique, and the absence of a requirement for costly *in vivo* metabolic ra-diolabelings. For these reasons it is an ideal way to characterize complex mixtures of antigens from biopsies of animal tissues.

Electrophoretic transfer coupled with the sensitivity of antibody–IPA illumination makes the Western blot a suitable method for analysis of products synthesized *in vitro* by messenger-dependent cell lysates (25). Rather than relying upon the incorporation of expensive translation-grade radiolabeled amino acids and the individual immunopre-cipitation reactions for detection of syn-thetic polypeptides, it is possible to visual-ize these products *in situ* on a gel blot with specific antibody and IPA. *In vitro* transla-tions of approximately 1 μg of MuLV ge-nomic RNA resulted in sufficient Pr65^{gag} to be detected after 5–6 h of direct radio-graphic exposure following antibody–IPA treatment of a gel blot; parallel translation with incorporation of [^{35}S]methionine al-lowed comparable visualization of Pr65^{gag} after overnight exposure (12–16 h) of a fluorographed gel. Western blotting has also been applied to studies concerned with the segregation of mRNA molecules and subsequent compartmentalized protein synthesis on free and membrane-bound polyribosomes. In this regard, certain nas-cent virus-specific polypeptides associated
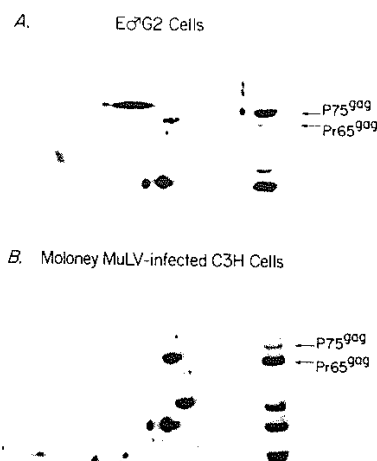

FIG. 5. Western blot and antibody–IPA detection of intracellular MuLV-specific antigens fractionated by 2-DGE. Lysates containing either 3 × 10^5 E♂G2 cells or 1 × 10^5 Moloney-C3H cells were first fractionated by cathode-directed isotachophoresis in cylindrical polyacrylamide–urea gels containing a pH 3.5–10 am-pholyte. Following isotachophoresis for 3000 V · h, the gels were electrophoresed at right angles in 10% polyacrylamide–SDS slab gels. The slab gels were subjected to electrophoretic blotting and the blots sub-sequently reacted with rabbit anti-p30 serum and IPA as described in the text. Radiographic exposure time was 30 min. In both panels the direction of isotacho-phoresis was from right to left. Marker lanes to the right on the second dimension gels show the authentic samples run in a single dimension. A, E♂G2 cell ly-sate; B, Moloney-C3H cell lysate.

with such purified polysome preparations from infected cells have been detected by antibody–IPA illumination on nitrocellu-lose blots.

Problems Encountered in Western Blotting

Distortion due to unequal volume expan-sion of the gel and the nitrocellulose may arise during long-term electrophoresis, par-ticularly at high accelerating voltages. This may lead to diffuse bands (in SDS gel elec-trophoresis) or spots (in 2-DGE). Joule heat-ing can be minimized by keeping voltages below 10 V/cm. Further swelling and shrinking can be prevented by empirically

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matching the methanolic composition of the electrode solution with the amount of crosslinking in the polyacrylamide matrix. Gels prepared from a stock solution of 30% (w/w) acrylamide and 0.8% (w/w) bisacrylamide undergo no significant volume changes in the electrode solution described. Gels constructed with different matrix ratios may require slight modification in the methanol concentration or short (less than 1 h) preswelling in electrode solution to inhibit distortion. Failure to have good initial contact between the gel and nitrocellulose can lead to "skewing" of certain bands; this artifact can be seen with the bovine albumin and ovalbumin markers in Fig. 2.

A disadvantage of the Western blot from SDS–gels is the tendency of antigenically reactive sites (epitopes) on the fractionated molecules to be irreversibly denatured by the detergent (26,27). Although not generally a problem when utilizing polyvalent monospecific antisera for detection, this limitation is quite severe when screening panels of monoclonal antibodies. Since such antibodies are directed against single epitopes within an antigenic molecule (28), there is a likelihood that the reaction of interest may be abolished by the denaturing effect of the detergent. Therefore, when selecting monoclonal antibodies for use in Western blotting analysis it is necessary to test the SDS sensitivity of the specific epitopes. Although it is possible to achieve a greater degree of protein renaturation by substitution of various commercial grades of SDS in the SDS electophoretic electrode buffer ((29,30), unpublished observation), it is best to avoid this detergent altogether if suitable fractionation of sensitive antigens can be accomplished by other gel methods (e.g., slab gel isoelectric focusing).

Unlike blotting to diazotized paper (1,2,6), the radiolabeled probes of Western blotting are not readily removed from nonderivatized nitrocellulose for reuse of the replicas with different probes. Treatment of blots with detergent (5% SDS), chaotropic

agent (3 M NaSCN), or acid (pH 3) fails to preferentially remove probe and background radioactivity relative to the desorption of immobilized antigens.

CONCLUSIONS

The technique of Western blotting should prove valuable for the analysis of proteins fractionated on the basis of molecular weight in SDS gel electrophoresis. Use of unmodified nitrocellulose sheets and an electrophoretic mode of transfer provide speed and a simplicity of technique that, combined with essentially complete and quantitative protein transfer, is not achievable by other blotting methods. Coupled with antigen detection by antibody and IPA, the Western blot is a very sensitive method for visualizing specific proteins in complex antigenic mixtures. The use of radioiodinated protein A allows such analyses to be accomplished from cells in culture as well as from necropsied animal organs and biopsied tissues without having to resort to metabolic radiolabeling of the cellular antigens (N. Burnette, R. Tao, and R. Nowinski, unpublished). The blotting method can also be adapted to a large variety of other problems employing SDS gel electrophoresis for analysis. These include, for example, two-dimensional gel fractionation of cellular components, analysis of products synthesized *in vitro* in response to exogenous messenger RNA, and studies of nucleic acid binding proteins using appropriate radiolabeled nucleic acid probes (5). Realizing the limitations imposed by SDS denaturation, investigations utilizing monoclonal antibodies for detection may be undertaken if these probes are first selected on the basis of their reactivity against detergent-insensitive epitopes.

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