A Sensitive Immunoblotting Method for Measuring Protein Synthesis Initiation Factor Levels in Lysates of Escherichia coli*

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Protein synthesis initiation factor levels are measured in crude cell lysates of Escherichia coli MRE600 by use of a sensitive immunoblotting method. The method involves electrophoretic transfer of protein from sodium dodecyl sulfate-polyacrylamide gels onto nitrocellulose paper and subsequent incubation with a specific antisera and radioactive iodinated second antibody. The measurement of iodinated antibody attached to known amounts of initiation factor is determined by densitometric scanning of autoradiographs or counting radioactivity in excised protein bands. Linear standard curves over the range 1 to 300 ng of factor are obtained by these methods. Unknown amounts of initiation factor in crude cell lysates are measured accurately; values agree with previous radioimmune assay data. The immunoblotting method serves as an alternative to the radioimmune assay in measuring small quantities of protein in complex mixtures. Immunoblotting enjoys three major advantages: it is simple and rapid to execute; it is sensitive; and it is capable of distinguishing multiple forms of the antigen which separate in the gel system employed.

Knowledge of the cellular levels of enzymes and other proteins is an important part of understanding their functional role. We have been studying the protein synthesis initiation factors from Escherichia coli and are interested in determining how factor levels change as the bacteria progress into different physiological states. Toward this goal we developed a radioimmune assay capable of measuring the levels of each of three initiation factors, IF1, IF2, and IF3, in crude cell lysates (1). The method is sensitive in the range 20 to 300 ng of factor, but is rather laborious and difficult to perform. It also fails to distinguish multiple forms of a factor such as IF2, which is known to occur as 18,000- and 90,000-dalton proteins (2, 3). To circumvent these difficulties, we recently utilized non-equilibrium pH gradient electrophoresis on polyacrylamide gels, followed by a second dimension gel in SDS (4), to separate and quantitate initiation factors. This method is more rapid than the RIA, and is capable of distinguishing different molecular weight or isoelectric forms of the factors, but it suffers from two problems. Some proteins are not well resolved from other unrelated proteins in the lysate, and quantitative entry of all proteins into the gel system is not always attained.

New methods for analyzing proteins have been described which combine polyacrylamide gel electrophoresis and immunological techniques. Towbin and co-workers (5) described a technique for electrotransferring proteins from an SDS-polyacrylamide slab gel to a nitrocellulose sheet. The presence of specific proteins are then identified by treating the nitrocellulose with specific antibody. The presence of attached antibodies is determined by a coupled reagent which generates a color in situ. Renant et al. (6) transferred protein from gels to diazobenzyloxymethyl paper and identified specific antigens with antiserum followed by treatment with 125I-labeled protein A from Staphylococcus aureus. We have adapted these techniques to enable us to quantitate the levels of the initiation factors in crude cell lysates. The method is sensitive in measuring nanogram amounts of protein and is both simple and rapid. Its usefulness can be extended to quantitating changes in molecular weight or isoelectric forms for any protein for which specific antisera are available. While this work was in progress, a method was reported (7) for quantitating the subunits of the yeast cytochrome bc complex; it is similar to that described here, but is much less sensitive.

EXPERIMENTAL PROCEDURES

Materials—E. coli strain MRE600 cells were grown in morpholinopropanesulphonic acid-minimal media supplemented with 0.4% glucose, 1% Bacto-tryptone, and 0.5% yeast extract (8). The cells were harvested in exponential phase, sonicated in buffer (10 mM Tris-HCl, pH 7.4, 10 mM Mg acetate, 150 mM KCl) and clarified by centrifugation for 20 min at 30,000 × g. Protein concentration was determined by the methods of Lowry et al. (9) and Schaffner and Weissmann (10) with bovine serum albumin as standard. Initiation factors IF1, IF2a, and IF3 were prepared as described (2) and were greater than 95% pure. Preparation of rabbit antiserum against each initiation factor has been described previously (1). To obtain purified immunoglobulin G, crude antiserum (500 μl) against IF2a was dialyzed against 1 liter of buffer (20 mM K phosphate, pH 8.0, and 0.01% sodium azide), and passed through a 1-ml Affi-Gel blue (Bio-Rad) column equilibrated with the same buffer. The flow-through fractions containing IgG were pooled and the protein was precipitated by adding ammonium sulfate to 45% saturation. The precipitated protein was collected by centrifugation and resuspended in 200 μl of PBS (10 mM K phosphate, pH 7.2, 150 mM NaCl) and dialyzed against PBS. Purified anti-IF2a IgG and S. aureus protein A (Sigma) were radioiodinated (11). Specific activities were between 0.5 and 1.5 × 10^6 cpm/μg.

Electrotransfer of Protein to Nitrocellulose—Slab gel electrophoresis (10% acrylamide) was conducted by use of the SDS-PAGE system of Laemmli (12). Upon completion of the electrophoretic run, protein was transferred to nitrocellulose sheets essentially by the method of Towbin et al. (5). Slab gels (10 × 14 cm) were placed onto wetted nitrocellulose paper supported by Whatman 3MM paper and scouring pads on each side. The sandwich was placed between two 3-inch plastic sheets with 0.5-inch holes drilled into them opposite the position of the sandwich. The entire assembly was firmly wrapped with glass-fiber tape and placed between two vertical wire mesh electrodes encased in a plastic chamber. The assembly fit snugly into
the chamber to allow the current to pass only through the gel via the
0.5-inch holes. The chamber was filled with electrode buffer (25 mM
Tris-base 192 mM glycine, 20% methanol, at pH 8.3) and electropho-
resis was carried out at 20 V, 300 mA for 6 h with the anode on the
nitrocellulose side of the sandwich. The nitrocellulose paper was
stained with Amido black (0.1% in 45% methanol and 10% acetic acid),
destained (90% methanol and 2% acetic acid) and stored in water for
up to 1 week (10).
Antibody Incubation with Blotted Nitrocellulose—The blotted
and stained nitrocellulose papers were incubated with 3% bovine
serum albumin in saline (0.9% NaCl, 10 mM Tris-HCl, pH 7.4) for 1 h
at 37°C and rinsed twice with buffer A (0.2% SDS, 0.5% Triton X-
100, 0.5% bovine serum albumin, and 0.01% NaN₃ in saline) (13). Anti-
IF1 and anti-IF3 were diluted with buffer A 1:25 and 1:100, respect-
ively; 125I-anti-IF2a was used at 0.1 to 0.4 µg/ml. The diluted antise-
rum (10 to 40 ml) and blots were incubated together with gentle
shaking at room temperature for 12 h. The blots were washed with
buffer A for 30 min and five changes. When the second antibody,
125I-labeled S. aureus protein A, was used, it was diluted in buffer A to
5 × 10⁶ cpm/ml and 10 to 40 ml were incubated with the washed
blots for 6 h. Again the blot was washed with buffer A for 30 min and five
changes. After washing, the blots were air dried and exposed to Kodak
X-OMAT SB film as indicated in the figure legends.
Quantitation of Radioactivity on the Nitrocellulose Blots—Two
different methods were used to measure the amount of 125I-IgG or
125I-protein A bound to the nitrocellulose blot. In the first method,
the autoradiograph was scanned in a Cary 210 Spectrophotometer
(Varian) with a gel-scanning accessory. The spectrophotometer was
interfaced with a Hewlett Packard 9845A computer. The individual
lanes on the autoradiograph were scanned and analyzed with use of a
gel-scanning program written by Harry Matthews and modified by
Frank Zucker (Davis, CA). The second method involved placing the
exposed autoradiograph on top of the nitrocellulose paper and with
a scalpel blade cutting out the darkened band and the nitrocellulose
sheet beneath. The nitrocellulose chip was counted in a Tracer
Analytic model 1197 (Searle) γ counter.

RESULTS AND DISCUSSION

Quantitation by Immunoblotting
Towbin et al. (5) and Renart et al. (6) described procedures
for electrotransferring proteins separated by SDS-PAGE onto
nitrocellulose sheets or diazobenzyloxymethyl paper and iden-
tifying specific proteins by treating the sheet with antibodies.
We modified the general procedure to allow quantitation of
the levels of proteins in a crude mixture. The quantitative
assay is dependent on the following conditions: all of the
protein in the sample must enter the polyacrylamide gel and
the amount of antigen analyzed; and the presence of other
components in the sample may not affect the assay results.
These conditions are satisfied when assays with crude E. coli
lysates are analyzed as described under "Experimental Pro-
cedures." The following control experiments document this
assertion.

Conditions for Quantitative Transfer—We tested the rate
of electrotransfer of cell lysate proteins over 6 h by exchanging
the nitrocellulose blot with a new sheet every hour. Electro-
transfer of small proteins proceeds more rapidly than large
proteins, as shown in Fig. 1A. Low molecular weight proteins
required 1 to 2 h for quantitative transfer in our apparatus,
whereas the highest molecular weight proteins needed up to 6 h.
That all of the protein is transferred after 6 h was shown by
staining the polyacrylamide gel with a sensitive silver stain
(14); no residual proteins were detected in the gel (results not
shown). It is important to show that all of the proteins which
leave the gel actually bind to the nitrocellulose sheet. We
analyzed different amounts of lysates and pure IF2a by using
two nitrocellulose sheets and asked at what protein level is
the first nitrocellulose sheet saturated, resulting in binding to
the second sheet. As shown in Fig. 1, all of the protein binds
to the first sheet (panel B) when 10 µg of lysate are analyzed;
some smaller molecular weight proteins (range: 10,000 to
20,000) pass through to the second sheet (panel C) at 40 µg of
lysate; 100 µg of lysate substantially exceeds the capacity of
the first nitrocellulose sheet. IF2a as a single sharp band was
reported by Buhler et al. (13) and was treated with a 1:100 dilution of anti-IF3 and 125I-protein A. Lane 5 contains 40 µg of lysate
protein and was treated with a 1:25 dilution of anti-IF1 and 125I-protein A. The migration positions of standard initiation factors are shown on the right.

FIG. 1. Electrotransfers and immunoblots. Panel A, effect of
electrotransfer time. An E. coli lysate (40 µg of protein) was subjected to PAGE and electrotransfer as described under "Experimental Procedures." After each hour of electrotransfer, the nitrocellulose sheet was removed and replaced by a new sheet, and electrotransfer was continued. Each sheet was stained with Amido black and photog-
graphed. The figure shows photographs of the blotted, stained lane obtained after 1, 2, 3, 4, 5, and 6 h of electrotransfer. Panel B, E. coli
lysates (10, 40, and 100 µg of protein) were electrotransferred for 6 h in a sandwich containing two nitrocellulose sheets. The figure shows
the Amido black-stained sheet nearer the gels. Panel C, the same electrotransfer experiment as panel B, except that the stained sheet
shown is that further from the gel. Panel D, IF2a in nanogram amounts shown at the top of the figure were subjected to PAGE and
immunoblotting with 125I-anti-IF2a as described under "Experimental Procedures." The figure shows the autoradiograph exposed for 21 h.
Panel E, the figure shows autoradiographs of immunoblots of crude lysates of cells harvested in early exponential phase from rich media.

Lanes 1 to 3 contain 5, 10, and 20 µg of lysate protein, respectively, and were treated with 125I-anti-IF2a. Lane 4 contains 40 µg of lysate
protein and was treated with a 1:100 dilution of anti-IF3 and 125I-protein A. Lane 5 contains 40 µg of lysate protein and was treated
with a 1:25 dilution of anti-IF1 and 125I-protein A. The migration positions of standard initiation factors are shown on the right.
Procedures." This is a suitable method if the band to be cut out is well isolated from other labeled bands. The bands of 125I-anti-IF2 from Fig. 1D were excited and counted, and results are shown in Fig. 3 (curve a). This standard curve is linear over a range from 1 to 300 ng. In the second method, an autoradiograph of the blot was developed and scanned, and the peaks were integrated to yield areas which are proportional to the radioactivity present. A problem with using exposed x-ray film is that it may become saturated and no longer give a linear response to increased radioactivity. Fig. 2 shows that a plotted set of IF2a standards (30 to 300 ng) is exposed for longer lengths of time, intensity (area) of the bands in the exposed autoradiograph begins to level off. In order to produce an adequate standard curve, an exposure time which is in the linear range (i.e., parallel to the dashed line in Fig. 2) of all the standard points must be chosen. Such a standard curve, developed from an autoradiograph of the blot illustrated in Fig. 1D, is shown in Fig. 3 (curve b). As in the direct counting method, the standard curve is linear over a wide range. The scanning method is more ideally suited to analyzing a number of labeled bands in a single gel lane, especially if they are not well separated.

Effect of Lysate Components on Assay—An IF2a standard curve was determined in the presence of a constant amount of cell lysate. The same incremental increase in radioactivity in the IF2a bands, due to the addition of known amounts of IF2a, was observed whether lysate was present or not (results not shown). The amount of IF2a measured is therefore the sum of the amounts in the lysate and in the purified fractions added; no effect of one on the other was observed.

Initiation Factor Levels and Molecular Weight Forms in Cell Lysates

Bacterial cells were harvested in exponential phase and lysed into nondenaturing buffer as described under "Experimental Procedures." Analyses of such clarified lysates with antisera against IF1, IF2a, and IF3 are shown in Fig. 1E. These and similar autoradiographs were scanned in the gel region appropriate for each factor, and factor levels were computed from the standard curves shown in Fig. 3. The response for all factors is linearly proportional to the amount of lysate analyzed, as shown in Fig. 4. Levels for the three initiation factors are given in Table I.

The increases in lysates with anti-IF2a show a number of major bands, the highest molecular weight form corresponding to IF2a (Fig. 1E, lanes 1 to 3). When cells are lysed directly into SDS buffer, most of the other bands decreased in intensity and only two forms are seen, corresponding to IF2a and IF2b. To determine whether or not these other bands are partial proteolytic products of IF2a or are antigens recognized by the antiserum against IF2a has no significant effect on the other bands. Thus several factors are not well separated.

### Table I

Initiation factor levels in cell lysates

| Factor | RIA   | Blot  |
|--------|-------|-------|
| IF1    | 0.51  | 0.27  |
| IF2 (total) | 4.4 | 25    |
| IF2a   | 1.5   | 1.89  |
| IF3    | 0.35  | 3.5   |
| IF3    | 9.21  | 3.5   |
| IF3    | 3.91  | 3.91  |

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levels of contaminating antibodies which bind to other bacterial proteins and that the proteins which bind to anti-IF2a are truly related to IF2b. The blots also show that the IF2b form which has been purified (2, 3) is present in intact cells and is not an artifact of isolation.

When lysates are analyzed with anti-IF3 (Fig. 1E, lane 4), a single major band is seen which corresponds in molecular weight to IF3. Only a single molecular weight form of IF3 is seen, whereas two forms of IF3 have been reported (15, 16) which differ in length by 6 amino acid residues (17). Apparently, the second form is not present in these extracts of strain MRE600. Anti-IF1 serum (Fig. 1E, lane 5) shows a major band corresponding to IF1 and a number of minor bands of higher molecular weight, presumably due to contaminating antibodies. The detection of contaminating antibodies in the anti-IF1 serum occurs in part because the titer of anti-IF1 antibodies is lower and more serum is used in the immunoblotting procedure. Nevertheless, the band corresponding to IF1 is the strongest in intensity, and quantitation of the factor is not compromised by the low level impurities in the antiserum. A detailed analysis of the various forms of the initiation factors and their degradation products will be presented elsewhere.6

Comparison with Other Methods

In earlier work, we developed a radioimmune assay which is able to measure amounts of the three initiation factors in crude cell lysates. When we compare the amounts of initiation factors in crude cell lysates from exponential and stationary cells as assayed by the radioimmune assay and the blotting assay, there is good agreement (Table I). The amounts of IF3 are nearly identical. The amounts of IF1 are one-half in the blotting assay. Because of the low antibody titer and low amount of IF1 (levels near the nonlinear portion of the RIA standard curve), the RIA analyses may have been inaccurate. A different problem exists with anti-IF2a. Fig. 1E shows the numerous lysate proteins which bind to anti-IF2a. When the amount of IF2a by the blotting assay is compared with the RIA, it is found to be one-half of the RIA. However, the numerous proteins which bind to anti-IF2a are probably related to IF2a. If all of the radioactivity between IF2a and IF2b are added together, a comparable number to the RIA figure is obtained (Table I).

The quantitative immunoblotting procedure compares favorably with the more classical radioimmune assay, and in some ways is superior. Both methods are comparably sensitive and can measure protein levels in complex mixtures. The RIA requires either monospecific antisera or a radioactive antigen of very high purity. Immunoblotting can tolerate considerable levels of antibody impurities if the antigens recognized are separable by polyacrylamide gel electrophoresis. Furthermore, immunoblotting can distinguish different molecular forms of an antigen whereas the RIA cannot. For example, in this work we are able to measure the IF2a and IF2b molecular weight forms independently. If isoelectric focusing gels rather than SDS-gels are used, forms differing in charge (e.g. phosphorylated forms) can be distinguished and their levels quantitated. A further advantage of the immunoblotting procedure is that antibody cross-reactivity with other proteins can be readily detected and need not influence the quantitative results. Thus, this method provides significant advantages over the RIA in some situations, and yet is comparably rapid and simple in execution. We are currently using the quantitative immunoblotting procedure to analyze initiation factor forms in mammalian cells and as a screening procedure for identifying recombinant DNA clones carrying bacterial initiation factor genes.

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