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Western Blotting of Histones from Acid–Urea–Triton- and Sodium Dodecyl Sulfate–Polyacrylamide Gels

JAKOB H. WATERBORG AND RODNEY E. HARRINGTON[1]

Department of Biochemistry, University of Nevada, Reno, Nevada 89557

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We have developed a method for histone transfer from acid–urea–Triton (AUT)-polyacrylamide gels to nitrocellulose filters which prevents the interference of Triton X-100 with the binding of histones to nitrocellulose. Equilibration of AUT gels in 50 mM acetic acid and 0.5% sodium dodecyl sulfate (SDS) allowed displacement of Triton by SDS without loss of band resolution. Electrotransfer of all histone species from treated AUT gels or from equilibrated SDS gels was complete within 1 h in a transfer buffer of Tris–glycine with SDS for increased transfer efficiency and methanol for histone binding. Nitrocellulose with a pore size of 0.2 μm was optimal for histone detection. © 1987 Academic Press, Inc.

KEY WORDS: Western blotting; nitrocellulose, histones; AUT gel; SDS gel.

Polyacrylamide gels with acetic acid, urea, and Triton X-100 detergent offer unsurpassed possibilities for separation of histones by species, by sequence variation, and by postsynthetic modifications such as acetylation and phosphorylation (1–5). Use of such acid–urea–Triton (AUT)[2] gels in immunological analysis and comparison of histone species has not been reported. When the procedure described by Bio-Rad for the electrotransfer from acid–urea gels (6) was used, irreproducible patterns of histone transfer were observed. When analysis showed that core histones complexed with Triton X-100 did not transfer properly, we developed a method that removes Triton from AUT gels and allows subsequent blotting of histones from the gel to nitrocellulose filters.

MATERIALS AND METHODS

Histone gel electrophoresis. SDS (sodium dodecyl sulfate)–polyacrylamide gels (18% acrylamide–0.09% bisacrylamide) were used as described by Thomas and Kornberg (7). Discontinuous AUT gels at 15% acrylamide, 0.1% bisacrylamide, 8 M urea, and 8 mM Triton X-100 were run as described before (2,8). Selected gel strips were stained immediately or equilibrated, transferred, and subsequently stained, as described below. Calf thymus histones were obtained from Worthington.

Electrotransfers were done in a Bio-Rad transblot cell with the gel centered 5 cm from both platinum electrodes. The acetic acid transfer, as described by Bio-Rad (6), consisted of equilibration of gel slices in 0.7% acetic acid for 2× 30 min and electrotransfer for 3 h at 60 V (300 mA) and 4°C.

Standardized transfer of histones from AUT gels. AUT separating gels were 1 mm thick, 18-cm wide, and 12 cm long and contained a 2-cm-long stacking gel. They were run for 6 h at 150–200 V. Gels were washed on a rotary shaker for 2× 30 min in 300 ml of 50 mM acetic acid–0.5% SDS and for 2× 30 min in 300 ml of transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.1% SDS). The nitrocellulose membrane (0.2-μm

[1] To whom correspondence should be addressed.

[2] Abbreviations used: AUT, acid–urea–Triton; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide.

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pore size, Schleicher & Schuell, BA83) in the transfer sandwich was positioned at the anode side of the gel and electrotransfer was for 60 min at a 300-mA constant current (50 V) at room temperature. The temperature of the transfer buffer did not increase by more than 5°C over ambient values. After transfer, nitrocellulose filters were washed twice in fresh 10 mM potassium phosphate buffer, pH 7.2, with 0.3% Tween 20 and were stained overnight in this buffer with 1 ml of Pelikan indian drawing ink per liter (9). Gels were stained overnight in 0.1% Coomassie brilliant blue R-250 in 7% acetic acid–20% methanol and were destained in 7% acetic acid–20% methanol.

RESULTS AND DISCUSSION

Electrotransfer of histones from AUT gels to nitrocellulose in acetic acid, according to the procedure recommended by Bio-Rad for basic proteins in native gels (6), showed abnormal patterns of transfer. Only the strongest histone gel bands were transferred and could be detected by staining of the nitrocellulose blot (Fig. 1C). Staining of the gel with Coomassie blue after transfer showed that minor protein bands and edges of major bands had not transferred (Fig. 1B). This transfer pattern was observed repeatedly after transfer ranging from 2 h up to 16 h. The extent of this characteristic decreased gradually over many hours upon extended equilibration but was accompanied by a loss of band resolution and band intensity due to diffusion. Apparently one or more gel constituents interfere with histone transfer.

Solutions of calf thymus histones were filtered over nitrocellulose in the presence of individual gel components, and histone retention was quantitated by staining of the filters (9) (results not shown). Triton X-100 inhibited histone binding to nitrocellulose membranes, probably due to complex formation between core histones and Triton (1). Known antagonists of this complex formation are SDS, urea, and CTAB (cetyltrimethylammonium bromide) (2). Addition of CTAB to a solution of histones prevented their retention by nitrocellulose. Addition of 2 to 8 M urea did not interfere with histone binding but could not alleviate the effect of Triton. Addition of SDS did not interfere with histone binding in the range of 0.05 to 1% and was effective in restoring histone binding when added in large molar excess over both histones and Triton.

Gel equilibration for 1 h in Tris–glycine (pH 8.3) transfer buffer for SDS gels (6), supplemented with 0.1% SDS and 20% methanol, and electrotransfer of the AUT gel for 1 h as though the gel was a regular SDS gel showed transfer of all histone species (Fig. 1E), but transfer was only partial (Fig. 1D). Without SDS in the transfer buffer, virtually no transfer was obtained. Without methanol, histone retention by the nitrocellulose was strongly reduced. Longer periods of gel equilibration in transfer buffer lead to band diffusion without accomplishing complete transfer.

Histone filtration experiments over nitrocellulose in acetate buffers ranging from 10 mM to 1.5 M, with pH values ranging from 3.5 to 7.0, suggested that acidic conditions at low ionic strength might facilitate displacement of Triton by SDS. Equilibration of AUT gels in 50 mM acetic acid with 0.5% SDS prevented histone band diffusion while displacing Triton, and complete transfer of all histones was possible within 1 h (Figs 2D and 2E), with extensive transfer in 15 min (Figs 2B and 2C). Longer transfer times did not affect the histone pattern on the nitrocellulose filter (Fig. 2G). With this gel equilibration protocol the AUT gel marker dye, methylene blue, is retained by the gel as a sharp band and is transferred to the filter, serving as a convenient marker for filter orientation and alignment, for instance, during analysis of the protein pattern with antibodies.

Trace amounts of histones, primarily of H2A species which have the highest affinity among the core histones for Triton (4), can

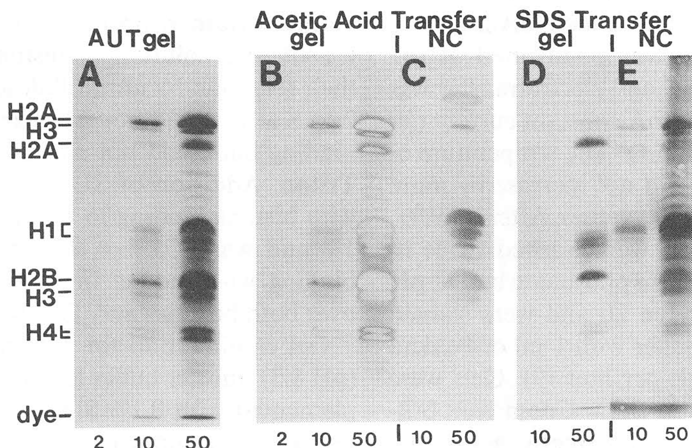

FIG. 1. Histone blotting from AUT gels. (A) Calf thymus histones (2, 10, and 50 μg of total histone per gel lane, as indicated) were electrophoresed in AUT gels and stained with Coomassie blue. The major histone bands and the marker dye, methylene blue, are marked. (B) Coomassie blue pattern of histones in the AUT gel after electrotransfer for 3 h in 0.7% acetic acid (Bio-Rad procedure). (C) India ink staining patterns of histones on nitrocellulose after transfer in 0.7% acetic acid. The pattern in (C) is longer than that in (B) due to gel expansion in 0.7% acetic acid relative to 7% acetic acid–20% methanol. (D) Coomassie blue pattern of histones in the AUT gel after gel equilibration for 1 h in transfer buffer (Tris–glycine–SDS–methanol) and electrotransfer for 1 h. (E) India ink pattern of histones on nitrocellulose after transfer in (D).

be detected on a second nitrocellulose membrane, positioned behind the first one during transfer (Fig. 2H). Longer transfer times lead to slightly stronger patterns on secondary filters, suggesting that histone binding to nitrocellulose is not completely stable. Slightly

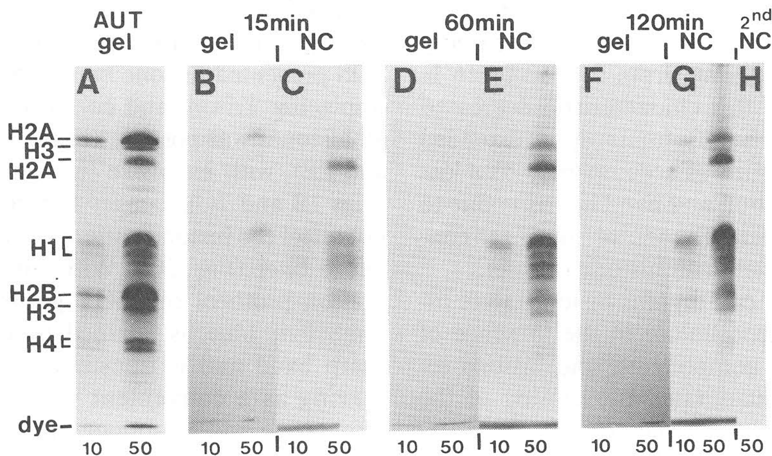

FIG. 2. Standard Western transfer of histones from AUT gel. (A) Calf thymus histone pattern in AUT gel stained with Coomassie blue (as in Fig. 1). Residual histone pattern in gels, stained with Coomassie blue, after electrotransfer for 15 min (B), 60 min (D), and 120 min (F). Histone pattern on nitrocellulose, stained with india ink, after electrotransfer for 15 min (C), 60 min (E), and 120 min (G). (H) India ink staining of a second nitrocellulose membrane after 120-min transfer.

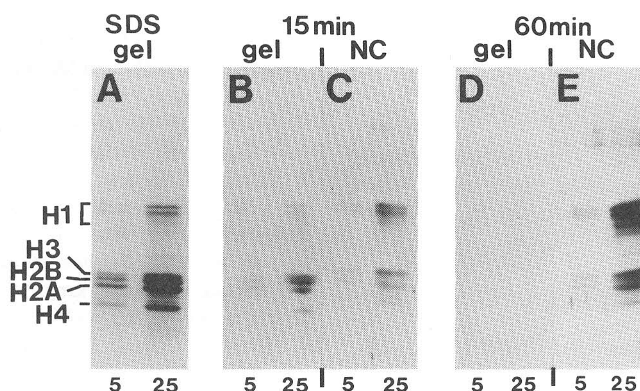

FIG. 3. Standard Western transfer of histones from SDS gel. (A) Pattern of calf thymus histones (5 and 25 μg total histone per gel lane) in SDS gel stained with Coomassie blue. The histone bands are marked. Residual histone pattern in gels stained with Coomassie blue after electrotransfer for 15 min (B) and 60 min (D). Histone pattern on nitrocellulose, stained with india ink after electrotransfer for 15 min (C) and 60 min (E).

stronger secondary histone patterns were also observed with membranes with a larger pore size (0.45 μm) while histones bound slightly more effectively to nitrocellulose of 0.1-μm pore size. Nitrocellulose membranes with pore sizes of 0.05 and 0.025 μm were unable to bind histones, as judged by india ink staining of the blots after transfer (results not shown). A marked increase in the amount of a histone species on the second filter was noted when more than 5 to 30 μg was present in a single band, suggesting that at these concentrations and depending upon the histone species the capacity of the nitrocellulose membrane to bind histones is exceeded.

Independent determination of the optimal parameters for the transfer of histones from SDS gels to nitrocellulose showed that the rate of histone transfer and the effect of membrane pore size on retention of and capacity for histones were identical to those obtained for AUT gels (Fig. 3). Gel equilibration in transfer buffer sufficed; equilibration in acetic acid–SDS was not required. The marker dye in this gel system, bromphenol blue, is lost from the gel during equilibration prior to transfer.

The observation that CTAB, like Triton, interferes with the binding of histones to nitrocellulose and the fact that SDS can also displace CTAB from its complex with histones (2) suggest that the method described for blotting of histones from AUT gels is also valid for acid–urea–CTAB gels that are used as a second-dimensional analysis for histones after AUT gel electrophoresis (2).

The sensitivity of histone detection on nitrocellulose by india ink staining (9) is somewhat less than that by staining of the histone bands in the gel by Coomassie blue, but it is much better than that by staining of the blots with amido black. The staining intensity varies among the histone species most easily seen in a transfer from SDS gels (compare Fig. 3A with Fig. 3E). The detection limits for single histone bands on 0.2-μm nitrocellulose, identical for transfers from AUT and SDS gels, range from approximately 0.2 μg for histone H1, 0.5 μg for histones H2A and H3, and 1.0 μg for histone H2B to 2.5 μg for histone H4. Thus histone detection by india ink appears to be less sensitive than detection of most other proteins (9).

The method described opens the way for immunological comparison among core histone variants separated in AUT gels, similar to published analyses for histone H1 variants separated in SDS gels (10,11). This may lead

to a better understanding of the function of core histone variants.

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