Nucleic Acids Research, 1993, Vol. 21, No. 15 **3591–3592**

Chemical Cleveland mapping: a rapid technique for characterization of crosslinked nucleic acid – protein complexes

Miranda Mirfakhrai and Alan M.Weiner*
Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510-8024, USA

Received June 10, 1993, Accepted June 17, 1993

Many RNA and DNA binding proteins can be crosslinked to nucleic acids by ultraviolet irradiation. Crosslinking is usually site-specific, so that label can be transferred from the nucleic acid to a specific peptide within the protein. Here we show that such a site-specifically labelled RNA binding protein can be rapidly and reliably characterized by treatment with the chemical reagent N-chlorosuccinimide (NCS) which cleaves the polypeptide backbone inefficiently but selectively at tryptophan residues (1, 2). The resulting array of labelled, partial peptide products serves as a fingerprint for the protein that directly contacts RNA or DNA. Using this NCS fingerprinting technique, it is possible to determine whether a potentially new RNA or DNA binding protein is related to a known protein, or whether the same nucleic acid binding protein is responsible for different bands in an electrophoretic mobility shift assay (EMSA). Additional advantages of the technique are that little if any purification of the protein is necessary, because crosslinking to labelled RNA or DNA usually proceeds efficiently in crude extract and the crosslinked products can be resolved by SDS–PAGE; crosslinking can also be performed in the gel after EMSA, allowing multiple shifted bands to be characterized in parallel (3); radiochemical quantities of the protein are sufficient for fingerprinting; and the cleavage reaction is performed in the gel without the inevitable loss of material accompanying elution.

The relatedness of two proteins can be determined by complete or partial peptide mapping using either enzymatic and chemical cleavage techniques (4, 5). The most popular of these techniques, often referred to as 'Cleveland mapping' (4), involves partial proteolysis with an enzyme like staphylococcal V8 protease, followed by denaturing gel electrophoresis to resolve the array of partial peptide products. Alternatively, the protein can be cleaved chemically with cyanogen bromide (CNBr), N-bromosuccinimide (NBS), tribromo-4-methylcyclohexadienone (TBS), or N-chlorosuccinimide (NCS) (1). Some of these reagents like CNBr (which cleaves at methionine) require special handling, while others like NBS (which cleaves at tryptophan, histidine, cystine and tyrosine) generate an overwhelming number of peptides. NCS, on the other hand, is innocuous and reliably generates distinctive partial peptide products (1, 2). Under acidic conditions in the presence of urea, NCS functions as a mild oxidant to induce cleavage at tryptophan residues through an oxindole intermediate; subsequent decomposition of the intermediate leads either to chain cleavage (with an efficiency ranging from 19% to 58%) or to modification of tryptophan without cleavage of the polypeptide chain. NCS usually does not cleave native proteins or aminoterminal tryptophan residues; methionine and cysteine are oxidized to methionine sulfoxide and cystine, but are not cleaved.

We suspected, based on amino acid sequence analysis, that the protein responsible for a site-specific ultraviolet crosslink to U6 snRNA was moesin, a membrane organizing extension spike protein (6). To confirm the identity of the protein in the crosslinked complex, uniformly labelled human U6 snRNA was generated by runoff transcription from a plasmid template using SP6 RNA polymerase. The labelled U6 transcript was added to a 40–65% ammonium sulfate fraction of HeLa nuclear extract and crosslinked for 5 minutes by 254 nm ultraviolet illumination (DNA Transfer Lamp; Fotodyne) in a 15 μl reaction containing 60% extract, 10 ng ^{32}P-U6 RNA (10^6 cpm), 250 mM KCl, 1 mM DTT, and 2 μg tRNA. After digestion with 1 mg/ml of RNase A at 37°C for 1 hour to trim crosslinked RNA and degrade uncrosslinked RNA, the crosslinked complexes were separated

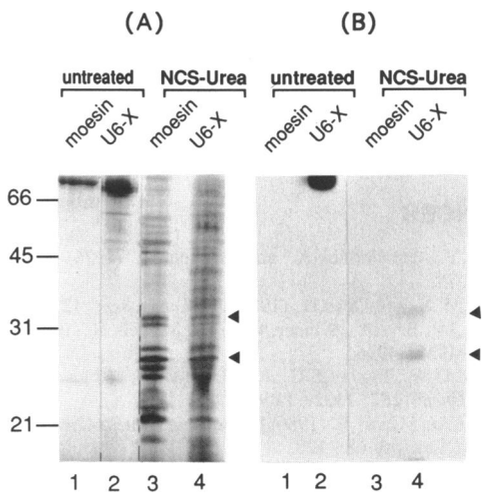

Figure 1.

* To whom correspondence should be addressed

on a 1 mm thick 10% SDS−PAGE (7). The labelled crosslinked U6-protein band was identified by autoradiography, excised, and then equilibrated with occasional agitation at room temperature in two 10 min washes with 25 ml H_2O followed by two 10 min washes with 10 ml 40% acetic acid, 6.6 M urea (Ultrapure; American Bioanalytical). After chemical cleavage for 30 min in 5 ml of the same solution containing 15 mM NCS, the gel band was washed twice in H_2O as before, equilibrated in three changes of 10 ml stacking buffer (10% glycerol, 15% β-mercaptoethanol, 3% SDS, 0.0625 M Tris−HCl, pH 6.8) for 20 min each, and then inserted directly into the well of a 5% stacking gel on a 15% SDS−PAGE. The gel pieces (originally 1 mm thick) can swell during the washes, so we recommend using a 1.5 mm thick gel for separation of proteolytic products. The peptide bands are then visualized by silver staining (8), thoroughly dried overnight to avoid cracking of the thick, high percentage gels, and exposed for autoradiography. As a control, purified moesin (0.3 μg) was resolved in parallel by SDS−PAGE, located by staining with Coomassie blue, and then excised and cleaved as for the labelled protein. Visualization of the partial NCS cleavage products by silver staining required no more than 0.2 μg of purified moesin.

The patterns of partial peptides generated by NCS cleavage of the nuclease digested crosslinked complex and unlabelled moesin are quite similar after silver staining (Figure 1A). The radiolabelled crosslinked complex also generates a visible NCS partial peptide pattern because moesin is an abundant cellular protein (6), and only a small fraction of the moesin is crosslinked to labelled U6 *in vitro* (Figure 1A, lane 4). An autoradiogram of the same gel (Figure 1B) indicates that the labelled NCS cleavage products of the crosslinked complex are a subset of those derived from moesin; the broadness of the labelled bands presumably reflects variable trimming of crosslinked U6 RNA. The NCS fingerprints are distinctive because partial cleavage by NCS yields multiple labelled peptides, even though the crosslink between moesin and U6 occurs at a single site (Mirfakhrai and Weiner, in preparation). The NCS cleavage patterns are also quite reproducible because the extent of partial cleavage with NCS is independent of protein concentration (1, 2). Uncleaved NCS-treated protein appears slightly larger than untreated protein, due to modification of tryptophan, methionine, and cysteine residues.

ACKNOWLEDGEMENT

This work was supported by grant GM31073 from the US National Institutes of Health.

REFERENCES

1. Shechter,Y., Patchornick,A. and Burstein,Y. (1976) *Biochemistry* **15**, 5071−5075.
2. Lischwe,M.A. and Ochs,D. (1982) *Anal. Biochem.* **127**, 453−457.
3. Williams,M., Brys,A., Weiner,A.M. and Maizels,N. (1992) *Nucleic Acids Res.* **20**, 4935−4936.
4. Cleveland,D.W., Fischer,S.G., Kirschner,M.W. and Laemmli,U.K. (1977) *J. Biol. Chem.* **252**, 1102−1106.
5. Fontana,A. and Gross,E. (1986) In *Practical Protein Chemistry*. John Wiley and Sons, Ltd. pp. 68−120.
6. Lankes,W.T. and Furthmayer,H. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8297−8301.
7. Laemmli,U.K. (1970) *Nature* **227**, 680.
8. Nielson,B.L. and Brown,L.R. (1984) *Anal. Biochem.* **141**, 311−335.