INCREASES IN PROTEIN A24 FOLLOWING TREATMENT OF EHRLICH ASCITES TUMOR CELLS WITH 1-METHYL-1-NITROSOUREA AND 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA

John M. DORNISH and Ingrid SMITH-KIELLAND
Department of Biochemistry, University of Oslo, Blindern, Oslo 3, Norway

Received 7 September 1981; revision received 29 October 1981

1. Introduction

Protein A24 is a conjugate of histone H2A and ubiquitin [1]. It is present in nucleosomes and represents ~2% of the histone content of core nucleosomes where it substitutes for H2A [2–4]. Decreases in protein A24 have been seen following thioacetamide treatment [5] or partial hepatectomy [6] and noted in transcriptionally active rat liver chromatin [7]. Protein A24 disappears from cells entering mitosis, thus revealing that ubiquitin must be released prior to chromatin condensation [8,9]. An enzymatic activity has been found that cleaves protein A24 into histone H2A and ubiquitin [10–12].

Here, acid-soluble nuclear proteins from Ehrlich ascites tumor cells treated with nitrosoureas have been investigated. In all experiments considerable changes in only one acid-extracted chromatin protein were found when analyzed by polyacrylamide gel electrophoresis. Due to its extraction in 0.4 N H_2SO_4, its M_r-value, position on two-dimensional gels, and elution characteristics on blue Sepharose, this protein has been identified as A24. Densiometric analysis of one-dimensional gels revealed that protein A24 increased in a dose-dependent manner following treatment of Ehrlich ascites cells with 1-methyl-1-nitrosourea and 1,3-bis(2-chloroethyl)-1-nitrosourea, even though protein synthesis was totally inhibited.

2. Materials and methods

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) was obtained from the Drug Research and Development Program (National Cancer Institute, National Institute of Health, Bethesda MD). 1-Methyl-1-nitrosourea (MNU) was purchased from ICN Pharmaceuticals. Ehrlich ascites tumor cells adapted to suspension culture were kept in exponential growth by daily dilution with Eagle's minimum essential medium with Earle's salts supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml benzyl penicillin, 100 μg/ml streptomycin, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.3) to 15 mM final conc. and non-essential amino acids. Cells were exposed to drugs for 1 h, at 37°C with appropriate controls. Nuclei from control and drug-treated cells were isolated by gentle hypotonic homogenization. After restoring isotonicity the remaining cytoplasm was removed with Triton X-100 in isotonic buffer [13]. Nuclear proteins were extracted twice with 0.4 N H_2SO_4 at ~5 × 10^7 nuclei/ml. Proteins were recovered by precipitation with 8 vol. acetone.

Polyacrylamide gel electrophoresis was carried out in SDS gels according to [14]. Two-dimensional gel electrophoresis was performed as in [15]. Acid-soluble nuclear proteins were chromatographed on blue Sepharose CL-6B (Pharmacia) as in [16] and fractions analyzed by one-dimensional gel electrophoresis as above. Gels were stained with Coomassie blue and scanned at 600 nm using a Gilford linear transport spectrophotometer.

3. Results and discussion

Fig.1 shows a one-dimensional SDS gel containing acid-soluble nuclear proteins from BCNU-treated Ehrlich ascites cells. The protein band marked A24 has M_r 27 000 and was barely visible in control cell

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

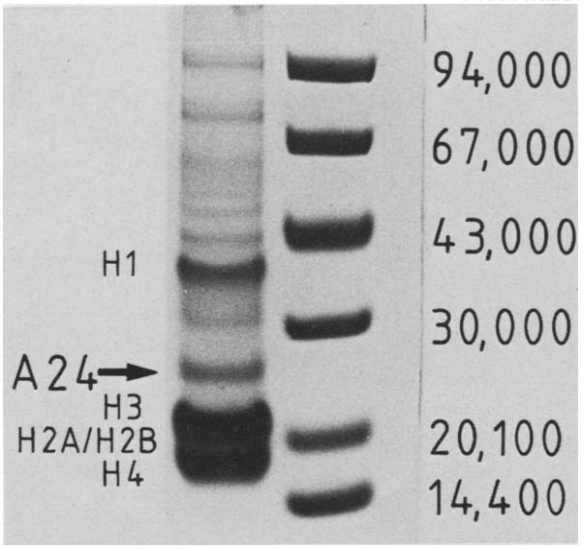

Fig.1. 7.5% polyacrylamide–SDS gel electrophoresis of 0.4 N H_2SO_4 soluble nuclear proteins. Electrophoresis was done as in [14]. Left: Ehrlich ascites tumor cells were incubated with 50 μg BCNU/ml for 1 h at 37°C. Right: Pharmacia low M_r calibration proteins; M_r-values are indicated.

preparations. This M_r for protein A24 agrees well with [2].

Increases in protein A24 following nitrosourea treatment are indicated in table 1. As can be seen, these increases in protein A24 relative to the sum of the core histones appear to be dose-dependent. Treatment of Ehrlich ascites cells with BCNU led to ~250% higher amounts of A24 than in control cells as judged by densiometric scanning while MNU treatment resulted in even larger increases. Table 1 also shows that A24 constituted 3.6% of the core histones in Ehrlich ascites cells, this value being somewhat higher than in [2,9].

Increases in protein A24 were also demonstrated by two-dimensional gel electrophoresis as illustrated in fig.2. The enlarged protein spot corresponding to the position of A24 in [2,3,15] seen after BCNU-treatment of cells (fig.2A) was not affected by the simultaneous presence of puromycin (20 μg/ml) during drug treatment (not shown). This indicates that protein synthesis cannot be responsible for the larger amounts of protein A24 seen following nitrosourea treatment.

Greater amounts of A24 from nitrosourea-treated cells were also detected when fractions of acid-soluble

Table 1
Increases in protein A24 following nitrosourea treatment

Drug treatment	Ratio A24/ core histones	% Control
Control	0.0366	100
BCNU (10 μg/ml)	0.0601	163
BCNU (25 μg/ml)	0.0762	208
BCNU (50 μg/ml)	0.0904	247
MNU (100 μg/ml)	0.0751	205
MNU (200 μg/ml)	0.1126	307

Ehrlich ascites cells were treated for 1 h at 37°C with the indicated concentrations of drugs. The nuclei were subsequently isolated and the acid-soluble proteins extracted with 0.4 N H_2SO_4. Proteins were separated on 15% polyacrylamide–SDS gels, stained with Coomassie brilliant blue, and densiometric scans made at 600 nm. The ratio of protein A24 to core histones (H2A, H2B, H3, H4) was determined by weighing the areas under the protein peaks. Results represent the average of 2 determinations

nuclear proteins chromatographed on blue Sepharose were analyzed by polyacrylamide gel electrophoresis. A24 consistently eluted together with histones H2A and H2B demonstrating that its affinity for blue Sepharose is identical with H2A and not affected by the isopeptide linkage to ubiquitin (not shown).

There are 3 possible ways in which nitrosoureas could cause the apparent increases in protein A24.

(1) Alkylation by BCNU and MNU could change the structure of protein A24 and prevent its cleavage mediated by A24 lyase. Another alkylating agent, nitrogen mustard, at up to 100 μg/ml does not induce increases in protein A24 (not shown). Any changes in protein A24 due to alkylation are thus not sufficient to affect its turnover.

(2) Crosslinking of DNA could prevent A24 cleavage. Since A24 is an integral part of nucleosomes, cross-linking of DNA by BCNU could hinder A24 from being cleaved by A24 lyase. However, results with nitrogen mustard (which is also able to cross-link DNA) do not support this reasoning. Furthermore MNU does not crosslink DNA but induces large amounts of A24 within the 1 h treatment period.

(3) There is the possibility that A24 lyase, the enzyme responsible for cleavage of protein A24, may be inhibited in nitrosourea-treated cells. Nitrosoureas decompose to form carbamoylating breakdown products [17,18]. The 2-chloroethyl isocyanate product of BCNU degradation has

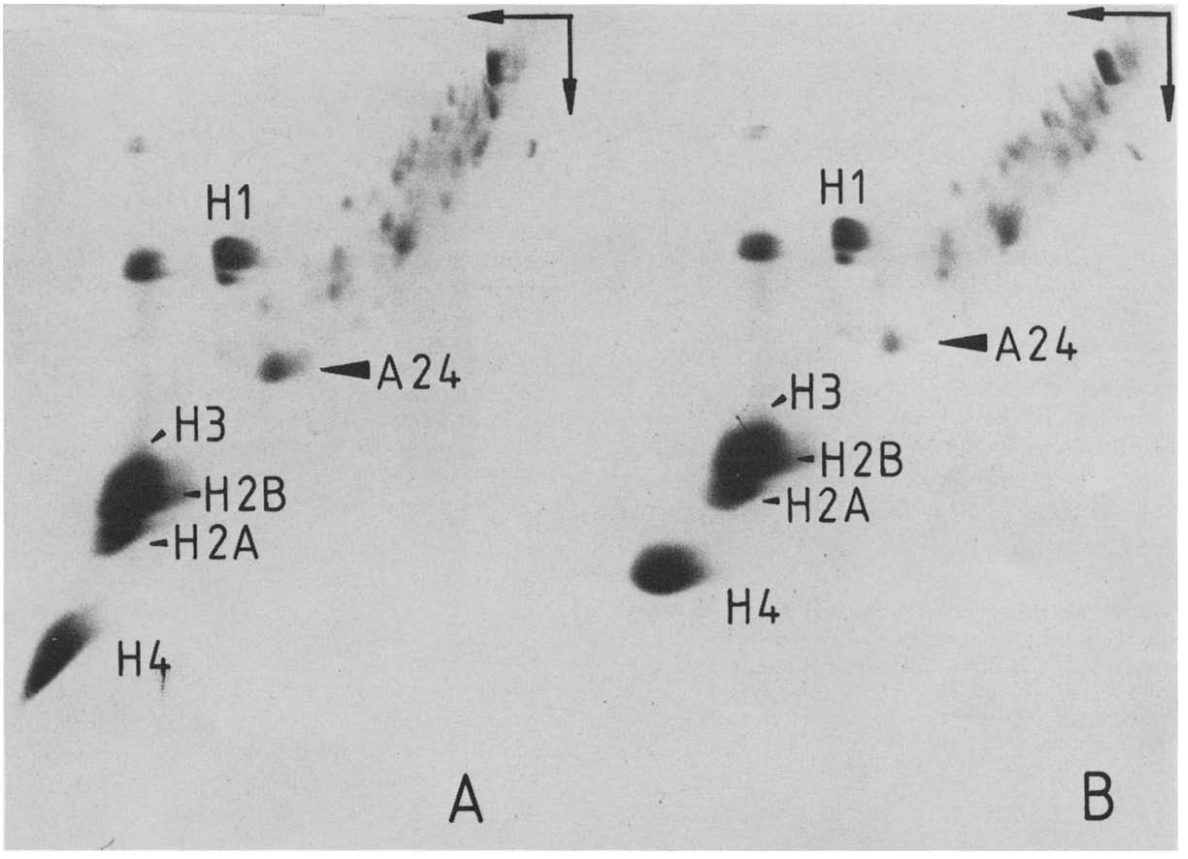

Fig.2. Two-dimensional polyacrylamide gel electrophoresis of 0.4 N H_2SO_4 soluble nuclear proteins from Ehrlich ascites tumor cells. The first dimension is acetic acid/urea (right-to-left) and the second is SDS (top-to-bottom) as in [15]. (A) Cells treated with 50 μg BCNU/ml for 1 h at 37°C; 120 μg protein applied. (B) Control cells, non-treated; 150 μg protein applied.

been shown to inhibit DNA polymerase [19,20] and ligase [21]. A very rapid turnover of ubiquitin–histone conjugates was shown in [22,23]. The formation and dissociation of A24 appears to be a fast process. Inhibition of the cleavage step by MNU or BCNU would therefore lead to a rapid increase in protein A24 and may represent a valid interpretation of these results.

The results presented here are of importance in that this is the first example of increases in protein A24. Treatment of cells with alkylating agents may help to elucidate the function of the cleavage enzyme A24 lyase. Furthermore, the action of alkylating agents on the structure of chromatin may have relevance to the further understanding of the rôle of A24 in nucleosome structure and function.

Acknowledgement

J. M. D. gratefully acknowledges the financial support of the Norwegian Research Council for Science and the Humanities (NAVF)

References

[1] Goldknopf, I. L. and Busch, H. (1977) Proc. Natl. Acad. Sci. USA 74, 864–868.
[2] Goldknopf, I. L., Taylor, C. W., Baum, R. M., Yeoman, L. C., Olson, M. O. J., Prestayko, A. W. and Busch, H. (1975) J. Biol. Chem. 250, 7182–7187.
[3] Goldknopf, I. L., French, M. F., Musso, R. and Busch, H. (1977) Proc. Natl. Acad. Sci. USA 74, 5492–5495.
[4] Martinson, H. G., True, R., Burch, J. B. E. and Kunkel, G. (1979) Proc. Natl. Acad. Sci. USA 76, 1030–1034.

[5] Ballal, N. R., Goldknopf, I. L., Goldberg, D. A. and Busch, H. (1974) Life Sci. 14, 1835–1845.
[6] Ballal, N. R., Kang, Y.-J., Olson, M. O. J. and Busch, H. (1975) J. Biol. Chem. 250, 5921–5925.
[7] Goldknopf, I. R., French, M. F., Daskal, Y. and Busch, H. (1978) Biochem. Biophys. Res. Commun. 84, 786–793.
[8] Matsui, S., Weinfeld, H. and Sandberg, A. A. (1979) Fed. Proc. FASEB 38, 491.
[9] Matsui, S., Seon, B. K. and Sandberg, A. A. (1979) Proc. Natl. Acad. Sci. USA 76, 6386–6390.
[10] Goldknopf, I. L., Cheng, S., Andersen, M. W. and Busch, H. (1981) Biochem. Biophys. Res. Commun. 100, 1464–1470.
[11] Andersen, M. W., Ballal, N. R., Goldknopf, I. L. and Busch, H. (1981) Biochemistry 20, 1100–1104.
[12] Goldknopf, I. L., Wilson, G., Ballal, N. R. and Busch, H. (1980) J. Biol. Chem. 255, 10555–10558.
[13] Krokan, H., Bjørklid, E. and Prydz, H. (1975) Biochemistry 14, 4227–4232.
[14] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[15] Orrick, L. R., Olson, M. O. J. and Busch, H. (1973) Proc. Natl. Acad. Sci. USA 70, 1316–1320.
[16] Kristensen, T. and Holtlund, J. (1980) J. Chromatogr. 192, 494–499.
[17] Wheeler, G. P., Bowdon, B. J. and Struck, R. F. (1975) Cancer Res. 35, 2974–2984.
[18] Cheng, C. J., Fujimura, S., Grunberger, D. and Weinstein, I. B. (1972) Cancer Res. 32, 22–37.
[19] Baril, B. B., Baril, E. R., Laszlo, J. and Wheeler, G. P. (1975) Cancer Res. 35, 1–5.
[20] Chuang, R. Y., Laszlo, J. and Keller, P. (1976) Biochim. Biophys. Acta 425, 463–468.
[21] Fornace, A. J. jr, Kohn, K. W. and Kann, H. E. jr (1978) Cancer Res. 38, 1064–1069.
[22] Seale, R. L. (1981) Nucleic Acids Res. 9, 3151–3158.
[23] Wu, R. S., Kohn, K. W. and Bonner, W. M. (1981) J. Biol. Chem. 256, 5916–5920.