Ultrastructural Analysis of v-*myb* Oncogene Product Cooperation with Components of Avian Cell Nuclear Matrix

(v-Myb oncoprotein / nuclear matrix / interaction / immunoelectron microscopy)

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Abstract. The cooperation of the v-Myb oncoprotein with extracted nuclear matrix of avian haematopoietic cells expressing the v-*myb* oncogene was studied by means of immunoelectron microscopy. The nuclear matrix was extracted by a gentle method of detergent treatment at moderate ionic strength and visualized either in ultrathin LR White sections, in unembedded resin-free sections, and in addition by the aqueous spreading technique. Using anti-Myb polyclonal antibody we have shown interaction of the v-Myb protein product with extracted nuclear matrix. This oncoprotein, however, was easily released from the structure by a detergent as well as by DNAase treatment and ammonium sulphate extraction. Prefixation of structures before detergent treatment prevented this extraction. The v-Myb protein marker was distributed in clusters or associated with fibrillar structures in most cases. Single markers decorating these fibrillar or less dense structures were also detected.

The v-Myb oncoprotein transforms myelomonocytic haematopoietic cells both *in vivo* and *in vitro* and causes leukaemia in avian myeloblastosis virus-infected birds (e.g. Graf, 1992; Lipsick, 1996). Previous studies have been directed preferentially to biochemical analysis of the v-*myb* oncogene and c-*myb* proto-oncogene functions (Klempnauer and Sippel, 1987; Klempnauer, 1988; Bartůněk et al., 1997) and they have shown that transformation by the v-*myb* oncogene product requires its nuclear localization and DNA-binding activity (Sheng-Ong, 1990). Myb proteins represent transcription factors containing an N-terminal DNA binding domain, a central transactivation domain and a C-terminal negative regulatory domain, which is substantially deleted in the v-Myb protein (Klempnauer et al., 1982; Weston and Bishop, 1989; Kalkbrenner et al., 1990). The c-*myb* proto-oncogene and the v-*myb* oncogene belong to the genes the protein products of which reside in the cell nucleus (Boyle et al., 1984; Klempnauer et al., 1984). However, data published on the subcellular localization of Myb proteins and their interactions with cellular structures are rather controversial. Their association with chromatin and nuclear matrix depending on experimental conditions has been described (Evan and Hancock, 1985; Klempnauer, 1988). On the other hand, it is believed that a large fraction of this protein is probably associated with nuclear matrix under physiological conditions (Shen-Ong, 1990). Recently, we have described, by means of immunoelectron microscopy, interaction of the v-Myb oncoprotein with spread chromatin of avian LSCC-BM2 cells expressing the v-*myb* oncogene (Korb et al., 1999). This analysis confirmed direct interaction of this protein with the chromatin structure; however, it was affected by detergent treatment.

In the present study we concentrated on immunoelectron microscopical analysis of v-Myb oncoprotein interaction with the nuclear matrix of LSCC-BM2 cells. As it was proposed that v-Myb protein association with nuclear matrix may be an experimental artifact (Evan and Hancock, 1985; Klempnauer, 1988), we used a gentle, highly reproducible method for extraction of this matrix (Fey et al., 1986), which seems to be insensitive to a small variation in the extracting procedure.

Material and Methods

Cells

The line of avian haematopoietic cells LSCC-BM2 expressing the v-*myb* gene (Moscovici et al., 1982) was grown in Dulbecco's modification of Eagle's medium containing 8% foetal calf serum (Sigma, St. Louis, MO), 2% chicken serum (Sigma, St. Louis, MO) and antibiotics (penicillin and streptomycin).

Nucler matrix preparation

Nuclear matrix was prepared from cells extracted by the method described by Fey (Fey et al., 1986), slightly modified: non-adhering BM2 cells were washed with phosphate-buffered saline (PBS), embedded in 0.5% agarose (type VII, Sigma, St. Louis, MO) and incubated in cytoskeleton buffer (CSK) (100 mM NaCl, 300 mM sucrose, 10 mM Pipes [pH 6.8], 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM PMSF, 2 mM vanadyl adenosine and 0.5% Triton X-100) at 2° C for 3 min. The suspension

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Abbreviations: BSA – bovine serum albumin, PBS – phosphate-buffered saline, PMSF – phenylmethylsulphonylfluoride.

was centrifuged at 800 *g* for 5 min and supernatant was discarded. The chromatin fraction was removed in modified CSK buffer (where 100 mM NaCl was replaced by 50 mM NaCl) by digestion with 100 µg/ml of RNAase-free DNAase I (Boehringer, Mannheim, Germany) at room temperature for 30 min. Ammonium sulphate was added from 1 M stock solution to a final concentration of 0.25 M and nuclear matrix was pelleted at 1000 *g* for 5 min, leaving the digested chromatin fraction in the supernatant. In some cases the cells were prefixed with 4% paraformaldehyde when embedded in agarose, prior to extraction.

Electron microscopy

Nuclear matrix structures were fixed in 4% paraformaldehyde in CSK buffer for 30 min at 4°C. The fixed structures were washed three times in 0.1 M cacodylate buffer, pH 7.2, at 4°C and dehydrated through a series of increasing ethanol concentrations that ended with three changes of 100% ethanol. The material was embedded at −20°C in LR White resin or treated by n-butyl alcohol and transferred to diethylene glycol distearate (DGD) (Polysciences Inc., Warington, PA) as described by Fey (Fey et al., 1986). Cutting, removing of DGD and critical point drying were performed by the procedure according to these authors.

Nuclear matrix was also analysed by the aqueous spreading technique (Berezny, 1991), which was modified. Briefly, 20 µl of hypophase containing 0.5 M ammonium acetate buffer with 1 mM EDTA, pH 7.5, the sample (free extracted cells not embedded in agarose) and cytochrome C (Sigma, St. Louis, MO, 1.12 mg/ml) were spread on the surface of double-distilled water. The surface film was picked up on electron microscopic grids, stained with uranyl acetate and rotary shadowed by Pt-Pd.

The samples were observed in a JEOL JEM 1200EX electron microscope.

Immunoelectron microscopy

Nuclear matrix embedded, spread or in resinless sections was pretreated for 30 min on drops of 0.5 mM ammonium chloride, followed by incubation on drops of blocking solution containing 1% BSA and 5% normal goat serum in PBS. The v-Myb oncoprotein was detected by immunostaining with primary anti-v-Myb antibody (rabbit, polyclonal, recognizing 19 amino acids of v-Myb and c-Myb protein products, which was obtained from Dr. M. Dvořák of the Institute of Molecular Genetics).

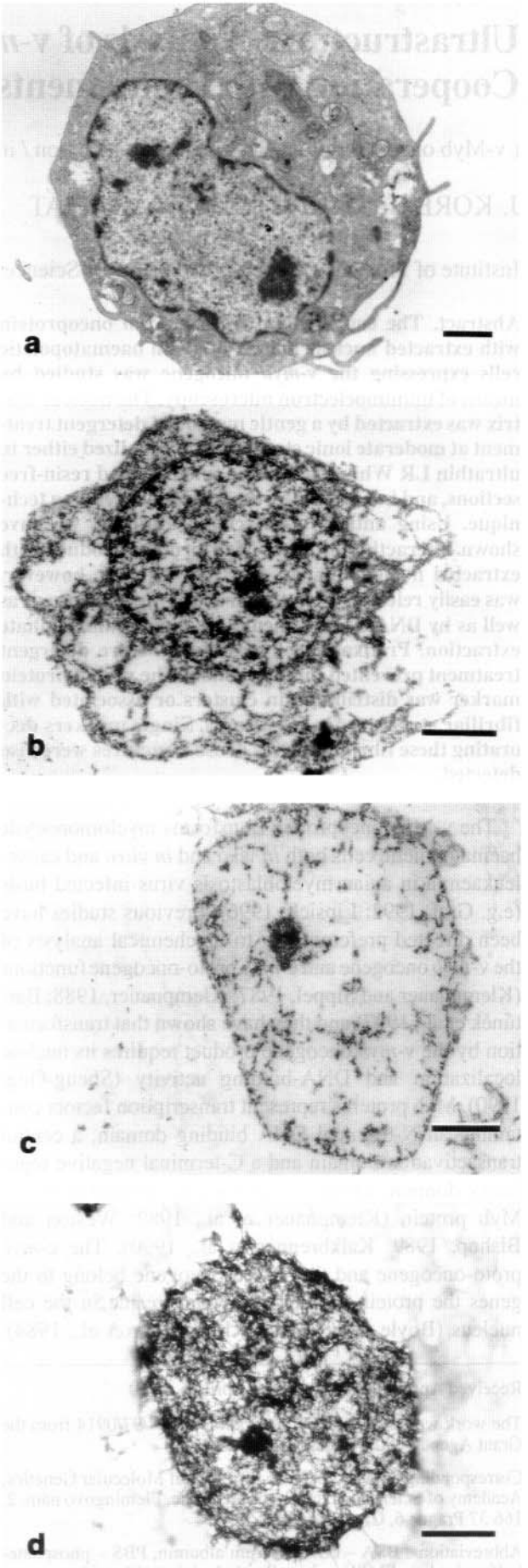

Fig. 1. Transmission electron micrographs of LSCC-BM2 cells. (a) Untreated control cells embedded in LR White resin, (b) cells extracted with Triton X-100 and embedded in LR White resin, (c) cells extracted with Triton X-100, digested with DNAase I and embedded in LR White resin, (d) cells extracted with Triton X-100, digested with DNAase I. Unembedded resin-free sections. Bars: 1 µm