INTERACTION OF V-MYB ONCOPROTEIN WITH SPREAD CHROMATIN OF AVIAN HAEMATOPOIETIC CELLS

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Summary. – Interactions of v-Myb oncoprotein with spread chromatin of avian LSCC-BM2 cells expressing *v-myb* oncogene were studied by means of immunoelectron microscopy. The application of this technique using anti-Myb polyclonal antibody combined with the Miller type spreading for visualisation of chromatin revealed the presence of Myb protein on stretched chromatin fibres. Intense labelling was apparent on the chromatin dispersed by hypotonic treatment, where the label was present frequently in clusters, although individual marks along the fibrillar molecules were also found. The combination of hypotonic and detergent treatment resulted in better dispersal of chromatin, more frequent detection of active transcription units, but also in removal of some proteins from chromatin fibres. The labelling of chromatin with anti-Myb antibody was substantially reduced in this case and was dependent on detergent concentration used. The marker was found less frequently on chromatin fibres usually present in clusters on remaining protein structures. Our findings confirmed direct interaction of v-Myb protein with chromatin structure. This interaction is apparently affected by detergent treatment.

Key words: v-Myb protein; spread chromatin; protein-chromatin interaction; avian cells; immunoelectron microscopy

Introduction

The avian myeloblastosis virus (AMV) *v-myb* oncogene representing a truncated and point-mutated version of the cellular proto-oncogene *c-myb* (Lipsick and Baluda 1986; Shen-Ong, 1990) transforms myelomonocytic haematopoietic cells both *in vivo* and *in vitro* and causes leukaemia in virus-infected birds (Graf, 1992; Introna *et al.*, 1994; Lipsick, 1996). Previous studies have shown that transformation by *v-myb* oncogene product requires its nuclear location and DNA binding activity (Shen-Ong, 1990). The protein products of the *v-myb* oncogene and *c-myb* proto-oncogene possess the characteristics of transcription factors that contain a N-terminal DNA binding domain, a central transactivation domain and a C-terminal regulatory domain, which is partly deleted in v-Myb protein (Klempnauer and Sippel, 1987; Weston and Bishop, 1989; Kalkbrenner *et al.*, 1990). c-Myb and v-Myb proteins contain leucine repeats that resemble leucine zipper structures of b-ZIP proteins (Hurst, 1995). *In vitro* binding studies demonstrated association of these leucine zipper structures with nuclear proteins of haematopoietic and HeLa cells (Kanei-Ishii *et al.*, 1992; Favier and Gonda, 1994). Studies of the interaction of Myb proteins with different subcellular structures represent one of the approaches for the understanding of their functional role. It is well known that *c-myb* proto-oncogene and *v-myb* oncogene belong to the genes, the products of which reside in the cell nucleus (Boyle *et al.*, 1984; Klempnauer *et al.*, 1984). However, published data on the subcellular location of Myb proteins are rather controversial. Their association with chromatin and nuclear matrix was described depending on experimental conditions (Evan and Hancock, 1985; Klempnauer, 1988). Our data recently obtained at the

Abbreviations: AMV = avian myeloblastosis virus; BSA = bovine serum albumin; PBS = phosphate-buffered saline; DMEM = Dulbecco's Modification of Eagle's Medium

ultrastructural level of *v-myb* oncogene product localisation confirmed its presence in the nucleus of both insect and avian cells (Štokrová *et al.*, 1995; Korb *et al.*, 1996). Extensive changes in cell structure were particularly observed in the ultrastructure of nucleus, nucleolus and chromatin organisation, which suggest specific interaction of v-Myb protein with these structures. These data led us to the detailed ultrastructural analysis of mutual interactions of v-Myb protein and chromatin. To examine this question, localisation of v-Myb antigen has been done directly on preparations of spread chromatin after lysis of tissue culture cells and labelling with specific antibodies.

Material and Methods

Cells. The line of avian haematopoietic cells LSCC-BM2 expressing *v-myb* gene (Moscovici *et al.*, 1982) was grown in Dulbecco's Modification of Eagle's Medium (DMEM) containing 8% foetal calf serum, 2% chicken serum and antibiotics (penicillin and streptomycin). In some experiments, the cells were synchronised using isoleucine deprivation and aphidicoline technique (Waltz *et al.*, 1996).

Chromatin spreading. The cells in medium were gently centrifuged, twice washed and resuspended in phosphate-buffered saline (PBS). An aliquot of 3 x 10^6 cells was pelleted, briefly washed in 0.2 mmol/l EDTA pH 7.5 and lysed in one of the following ways:

a) The pellet was resuspended in 2 ml of „pH 9 water" (10 mmol/l sodium borate buffer pH 9.22 diluted with redistilled water to a final concentration of 0.1 mmol/l) and kept for 30 mins on ice.

b) The pellet was resuspended in 5 ml of 0.02% Joy (detergent) solution in „pH 9 water" (Joy was a gift of Dr. F. Marec, Institute of Entomology, Academy of Sciences of the Czech Republic, České Budějovice) and kept from 15 to 60 mins on ice.

c) The pellet was resuspended in 500 ml of „pH 9 water", and 500 ml of 0.4% Joy solution in „pH 9 water" was added dropwise under constant Vortex stirring. The lysate was left for 5 mins, subsequently diluted with 20 ml of „pH 9 water" and kept for 15 mins on ice.

All lysates were fixed by adding 1/10 volumes of 0.1 mol/l sucrose (RNAse-free, Sigma) with 10% formaldehyde. Aliquots of the final mixture were then layered onto formaldehyde (1%)-sucrose (0.1 mol/l, RNAse-free) cushion (pH 8.5) in special centrifugation chambers, on the bottom of which were placed parlodion- and carbon-coated (hydrophylised by glow discharge or UV treatment (Namork and Johansen, 1982)) electron microscope nickel grids. Spreading of chromatin was performed as described by Miller and Baken (1972) by centrifugation at 2,200 x g for 10 mins at 4°C. The grids were removed, immersed in 0.4% Photo-flo 600 solution for 30 secs and air-dried.

Immunoelectron microscopy. Spread chromatin deposited on nickel grids was pretreated for 30 mins on drops of 0.5 mol/l ammonium chloride. After incubation the grids were transferred on drops of the blocking solution containing 1% bovine serum albumin (BSA) and 5% normal goat serum prior to staining with primary antibody (anti-v-Myb antibody (rabbit, polyclonal) recognising v-Myb and c-Myb protein products (obtained from Dr. M. Dvořák of this institute)) diluted 1:50. After washing in PBS containing 1% BSA the grids were stained with secondary antibody (GAR G5, Agar Scientific) diluted 1:20 in PBS containing 1% BSA. The grids were rinsed with PBS, then with distilled water and stained with 1% phosphotungstic acid in 75% ethanol. In some cases immunolabelling was performed prior to the spreading of chromatin. In this case an aliquot of the cell lysate was mixed with primary antibody (ratio 1:50) and incubated for 30 mins. Afterwards GAR G5 was added (ratio 1:20) and the mixture was incubated for another 30 mins. Aliqouts of the incubation mixture were then layered on sucrose-formaldehyde cushion as described before. The samples were observed with a JEM 1200 EX electron microscope.

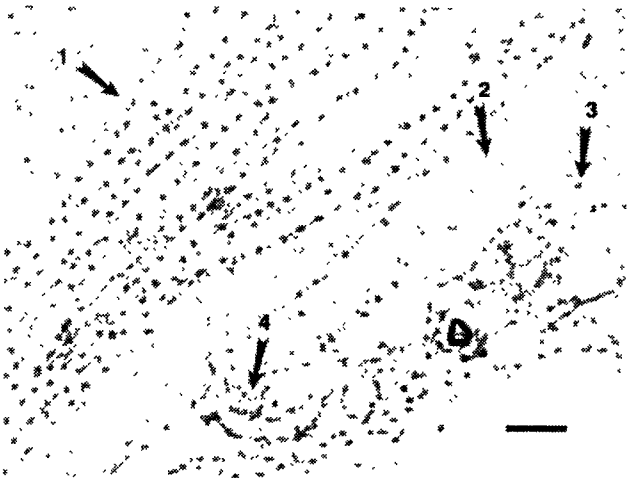

Fig. 1
Chromatin of LSCC BM-2 cells isolated by Miller procedure and contrasted by shadow-casting
Four structural motifs are apparent: (1) chromatin fibres with typical nucleosomal organisation, (2) thin strands free of particles, (3) thin fibres with sparsely attached particles, and (4) active transcription units. Bar = 0.1μm.

Results and Discussion

Hypotonic treatment of the cells in the absence of detergent resulted usually in rather poorly dispersed chromatin of avian LSCC-BM2 cells. However, even in this case a survey electron micrograph of spread preparations of chromatin revealed the presence of four different structural motifs: chromatin fibres with typical nucleosomal organisation („beads on a string"), thin strands free of particles, thin fibres with sparsely attached granular particles and active transcription units (Fig. 1). As the active transcription was only rarely detected in a nonsynchronised cell population, in some

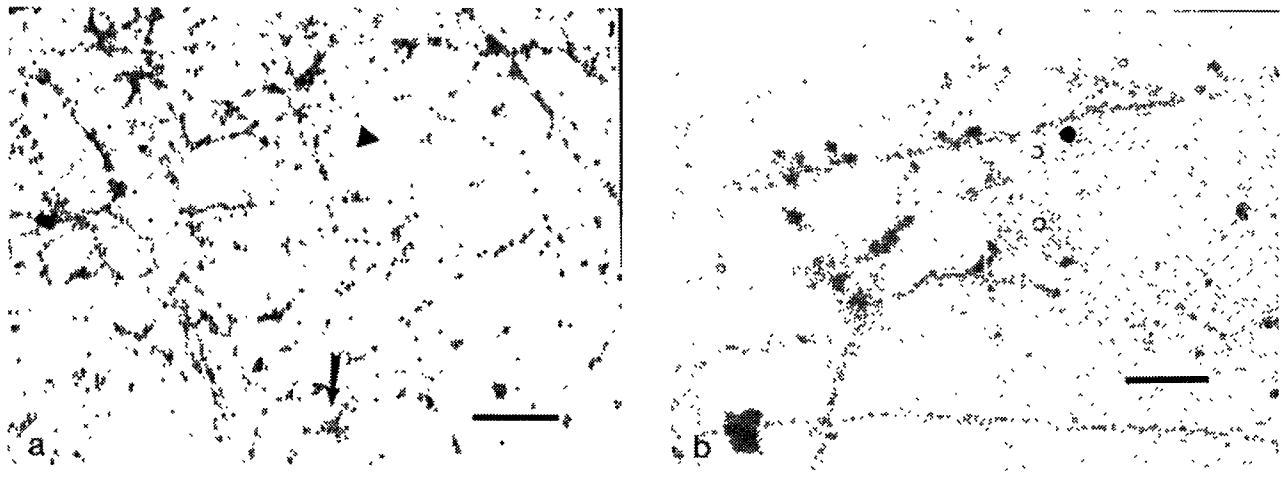

Fig. 2
Immunoelectron microscopic localisation of v-Myb protein on spread chromatin after hypotonic treatment
Cluster of markers (arrow) and individual marker (arrowhead). Experiment with (a) and without (b) primary antibody. Bars = 0.2 μm.

experiments a synchronised cell culture was used with the cells being in the middle of S phase.

The application of the immunoelectron microscopic technique using the anti-Myb polyclonal antibody combined with the Miller type of spreading (Miller and Beatty, 1969) after hypotonic treatment of cells revealed the presence of v-Myb protein marker on the stretched chromatin fibres (Fig. 2a). A relatively strong labelling was observed along rather poorly dispersed chromatin as well as on individual chromatin fibres. Most of the labelled chromatin was in transcriptionally inactive form, but the label was detected also in the proximity of transcription units. The label was found frequently in clusters (Fig. 2a, arrow), although individual marks along the fibrillar molecules were also present (Fig. 2a, arrowhead). It decorated predominantly the areas where protein structures attached to chromatin fibrils were apparent. The v-Myb antibody interacted with chromatin in both types of experiments either labelled prior to chromatin spreading or deposited already on parlodion-carbon membrane. In the latter case, however, an extensive blocking of free aldehyde groups by ammonium chloride was absolutely necessary for elimination of nonspecific binding. No labelling was detected in control experiments, where the primary antibody was omitted (Fig. 2b). The combination of hypotonic treatment with the action of detergent is recommended for a good dispersal of chromatin animal cells (Trendelenburg and Puvion-Dutileux, 1987; Fakan *et al.*, 1986). The use of the 0.02% Joy detergent in our experiments led to a much better dispersal of chromatin and to more frequently detected active transcription units. However, the labelling of chromatin with the anti-Myb antibody was substantially reduced (Fig. 3a). The Myb protein marker was found less frequently on chromatin fibres, usually detected in clusters present in this case on the remaining protein structures (Fig. 3a, arrows). These protein structures might represent some chromatin proteins resistant to the detergent treatment or the rest of some nuclear material (e.g. nuclear matrix) attached to chromatin. However, besides clusters of Myb protein marker individual markers were also occasionally observed on these protein structures (Fig. 3a, arrowheads). Control experiment where the primary antibody was omitted is shown in Fig. 3b.

When 0.2% Joy or Nonidet NP-40 were used for chromatin dispersal, the v-Myb protein binding to spread chromatin was only rarely detected (Fig. 4a, arrow). Although a good preservation of chromatin proteins using moderate concentrations of nonionic detergents was described (Hancock *et al.*, 1977; Fakan *et al.*, 1988), this approach in our hands resulted apparently in removal of some protein structures which bound v-Myb protein. On the other hand, we isolated morphologically intact nuclei from LSCC BM-2 cells even in the presence of these detergents. When primary antibody was omitted, no labelling on chromatin fibers was found (Fig. 4b).

The Myb protein association with chromatin and nuclear matrix was thought to be dependent on experimental conditions. It was proposed that its interaction with nuclear matrix may be an experimental artefact caused by irreversible precipitation of Myb proteins (Evan and Hancock, 1985) or that Myb proteins interact with nuclear matrix in a temperature-dependent manner (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 (for review see Shen-Ong, 1990).

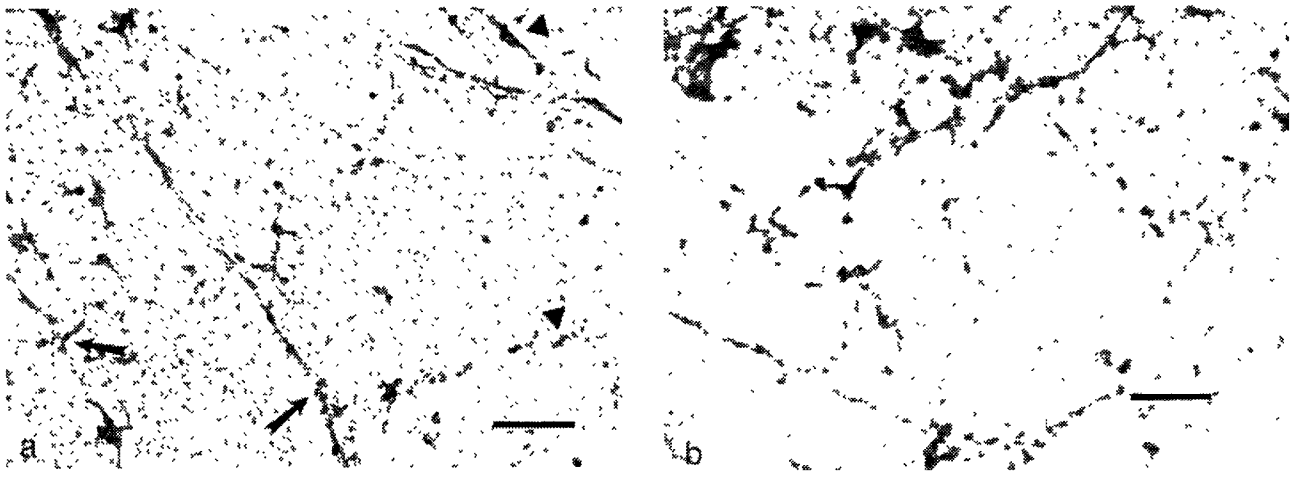

Fig. 3
Immunoelectron microscopic localisation of v-Myb protein on spread chromatin after treatment with detergent at low concentration (0.02% Joy)
Clusters of marker (arrows) and individual markers on remaining protein structures (arrowheads) Experiment with (a) and without (b) primary antibody. Bars = 0 2 μm.

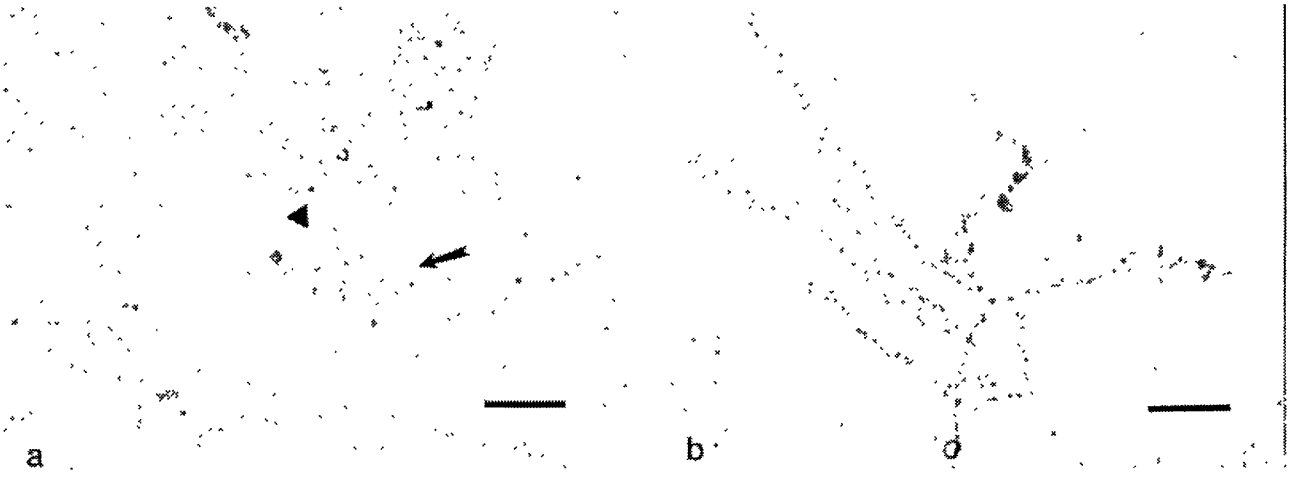

Fig. 4
Immunoelectron microscopic localisation of v-Myb protein on spread chromatin after treatment with detergent at high concentration (0.2% Joy)
Active transcription unit (arrow) and individual marker on chromatin (arrowhead). Experiment with (a) and without (b) primary antibody. Bars = 0.2 μm.

The immunolectron microscopical studies performed on spread chromatin directly revealed the interaction of v-Myb protein with this nuclear structure. This is in accord with our previous data on v-Myb protein localisation obtained at the ultrastructural level. This product was detected in interchromatin regions of the cell nucleus, usually associated with interchromatin granules and perichromatin areas (Korb *et al.*, 1996). Our findings presented in this paper confirmed direct interaction of v-Myb protein with chromatin structure. This interaction can be apparently affected by detergent treatment. Recently published data showed an *in vitro* interaction of v-Myb leucine zipper domain with two nuclear proteins (26 K and 28 K) of *v-myb*-transformed monoblasts (Bartůněk *et al.*, 1997). Since these proteins seem to be ubiquitously expressed (M. Dvořáková, personal communication), they may participate in the interaction of Myb with spread chromatin observed in this work.

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