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Subnuclear Associations of the v-*myb* Oncogene Product and Actin Are Dependent on Ionic Strength during Nuclear Isolation

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The method used to isolate nuclei has a direct effect on the subnuclear association of the v-*myb* product, $p48^{v\text{-}myb}$, and nuclear actin. Analysis of nuclei subjected to various isolation procedures showed that disruption of native nuclear structure during hypotonic treatment resulted in dissociation of $p48^{v\text{-}myb}$ from the nuclear matrix.

The product of the oncogene v-*myb* of avian myeloblastosis virus (AMV) in AMV-transformed myeloblasts is a 48,000-M_r nuclear protein, $p48^{v\text{-}myb}$ (4, 13). To help define the molecular role of $p48^{v\text{-}myb}$ in leukemogenesis, several investigators have sought to identify and define its nuclear interactions (1, 2, 8, 12, 14, 15). The association between $p48^{v\text{-}myb}$ and nuclear elements has been analyzed in fractionated nuclei isolated from AMV-transformed cells (BM2) by two different strategies (1, 8). One isolation procedure has been previously described (1, 3), in which nuclei are obtained from cells lysed under isotonic conditions in buffer containing 5 mM NaH_2PO_4 (pH 7.4), 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 2 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% Kyro EOB detergent (isotonic nuclei). After extraction of these isotonic nuclei with isotonic Nonidet P-40 (NP-40) buffer (0.5% NP-40, 10 mM NaH_2PO_4, pH 7.4, 120 mM NaCl, and 0.1 mM PMSF) (1), brief digestion with micrococcal nuclease at 37°C, and then extraction is 2.0 M NaCl (high salt), $p48^{v\text{-}myb}$ was removed from the nucleus in varying amounts in three distinct fractions: approximately 29% was contained in nucleoplasm, 7% was in nuclease-sensitive chromatin, and 64% was associated with the nuclear matrix/lamina complex (1). The other procedure we used to isolate nuclei relies on osmotic and shear force to separate the plasma membrane by disrupting cells which had been preincubated in hypotonic buffer (RSB: 10 mM NaCl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.4, 1.5 mM $MgCl_2$, 0.1% sodium deoxycholate [DOC], and 0.1 mM PMSF) with a Dounce homogenizer as described by Evan and Hancock (8).

After fractionation of these nuclei (hypotonic nuclei) by digestion with DNase I at low temperature (4°C) followed by extraction in high salt, most or all of $p48^{v\text{-}myb}$ was eluted from nuclei along with soluble nucleoplasm and chromatin (Fig. 1A) as previously described (8). However, when these hypotonic nuclei were transiently exposed to temperatures near 37°C during nuclease digestion, most or all of $p48^{v\text{-}myb}$ remained associated with the resulting insoluble nuclear complex (Fig. 1A) as reported previously (8). This apparent temperature effect on the nuclear association of $p48^{v\text{-}myb}$ indicates that the temperature during the treatment of nuclei isolated by this hypotonic procedure is an important factor in nuclear subfractionation. These results could suggest that localization of $p48^{v\text{-}myb}$ within the nuclear matrix fraction is an artifact induced by a temperature-dependent insolubilization of nuclear proteins.

Alternatively, the isolation procedure alone, not the subsequent method of subnuclear fractionation, may affect the associations of some nuclear proteins, including $p48^{v\text{-}myb}$. In nuclei isolated under hypotonic conditions the nuclear associations of $p48^{v\text{-}myb}$ might be different from its association in intact cells due to disruption of native nuclear structure (10, 11, 16). To test this hypothesis, we isolated isotonic nuclei from BM2 cells and used a temperature of either 4 or 37°C during nuclease digestion. If the association of $p48^{v\text{-}myb}$ with the nuclear matrix of nuclei isolated under isotonic conditions were to be detected only after elevation of the digestion temperature to 37°C, then this particular association would be a temperature-dependent artifact. This was not the case; whether the isotonic nuclei were digested with nuclease at 4 or 37°C, $p48^{v\text{-}myb}$ remained compartmentalized within the nucleus (Fig. 1B), as reported previously (1, 3).

The RSB extraction buffers used to prepare and wash crude hypotonic nuclei also contained 0.1% DOC, which has been shown to affect the solubility of nuclear components during fractionation of murine erythroleukemia cells (16). Therefore, for a rigorous comparison of the two isolation techniques, we added 0.1% DOC to our buffers and repeated the same nuclear fractionation procedure (Fig. 1B). DOC did not alter the fractionation profile of $p48^{v\text{-}myb}$. Conversely, the addition of both 0.5 mM $CaCl_2$ and 2.0 mM DTT to the RSB nuclear isolation buffer, which was present during the isolation and extraction of the isotonic nuclei, had no effect on the subnuclear localization of $p48^{v\text{-}myb}$ in hypotonic nuclei (not shown). These data indicate that the temperature effect on the nuclear associations of $p48^{v\text{-}myb}$ is not a generalized phenomenon, but appears to be restricted to nuclei isolated under hypotonic conditions.

Shown in Fig. 2 are light micrographs of nuclei obtained by the two different methods. The resulting isotonic nuclei retained the size and morphological features of nuclei present in intact cells, including an apparently normal nuclear envelope and prominent nucleoli (Fig. 2A and B). This is in agreement with what we have seen previously at the electron microscopic level (1). In contrast, the hypotonic nuclei isolated from these cells appeared to be physically altered, with a decreased size and loss of nucleolar structure (Fig. 2C and D). In addition, up to 40% of the hypotonic nuclei had

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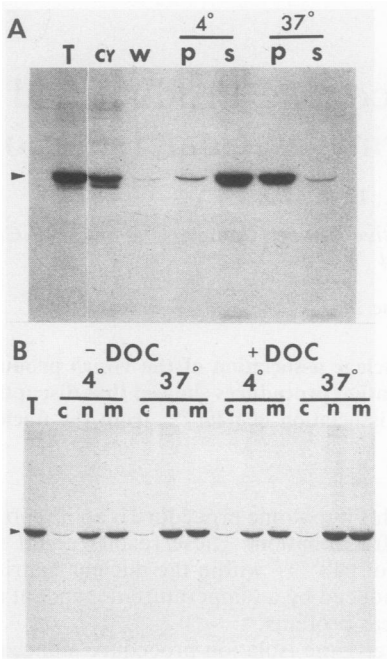

FIG. 1. (A) Temperature-dependent association of p48$^{v\text{-}myb}$ with the insoluble nuclear fraction of hypotonic nuclei. AMV-transformed myeloblasts (BM2) metabolically labeled with [^{35}S]methionine were washed in ice-cold RSB (8), swollen in ice-cold RSB for 10 min, and then broken with three to five strokes in a Dounce homogenizer. The nuclear fraction was pelleted by centrifugation at 1,000 × g at 4°C, the cytoplasmic supernatant was removed, and then the crude nuclear pellet was washed once in ice-cold RSB. The resulting nuclei were purified as described (8) and then digested with RSB-digitonin buffer containing DNase I at either 4 or 37°C (8). After digestion, 1 volume of high-salt buffer (8) was added, followed by a 60-min incubation on ice. The insoluble nuclear fraction was separated from solubilized chromatin by centrifugation, and each fraction was lysed by the addition of concentrated detergent buffer. Each fraction was adjusted to 10^7 nuclei or nuclear equivalents per ml and then immunoprecipitated with anti-*myb* peptide (P4) antiserum as described (1, 4). Immunoprecipitates were resolved by SDS-PAGE on a 10% polyacrylamide gel and then analyzed by fluorography. Lanes: T, whole cells; Cy, cytoplasmic supernatant; W, nuclear wash; P, insoluble nuclear fraction; S, soluble nuclear fractions. (B) Temperature-independent association of p48$^{v\text{-}myb}$ with the nuclear matrix/lamina fraction of isotonic nuclei. BM2 myeloblasts metabolically labeled with [^{35}S]methionine were washed in ice-cold Tris-glucose and then fractionated as previously described (1) into nucleoplasm, chromatin, or matrix/lamina in the presence or absence of 0.1% DOC. Nuclease digestion was carried out at either 4 or 37°C as indicated. Total cell and subnuclear lysates were adjusted to 10^7 cell equivalents per ml, immunoprecipitated and analyzed by SDS-PAGE. Lanes: T, total cells; c, chromatin fraction; n, nucleoplasmic function; m, matrix/lamina fraction.

released part of their chromatin content, as judged by light microscopy (not shown). Thus, hypotonic nuclei were visibly damaged by the isolation procedure prior to subfractionation.

Purified hypotonic nuclei (8) were then fractionated into nucleoplasm, chromatin, and nuclear matrix by the method we devised for subfractionating isotonic nuclei by resuspending them in kyro EOB buffer. We found that the association of a substantial fraction of p48$^{v\text{-}myb}$ with the nuclear matrix fraction occurred only at 37°C (Fig. 3, lanes m), whereas at 4°C p48$^{v\text{-}myb}$ appeared to be evenly dispersed between the soluble nucleoplasmic fraction (Fig. 3, lanes n) and chromatin fraction (Fig. 3, lanes ch). This indicates that the isolation of nuclei under hypotonic conditions was sufficient to disrupt the association of p48$^{v\text{-}myb}$ with the nuclear matrix and that hypotonic nuclei underwent a transition that was not reversed by incubation in isotonic buffer. Furthermore, nuclei isolated under our isotonic conditions and subsequently incubated in hypotonic buffer for 30 min also showed dissociation of p48$^{v\text{-}myb}$ from the nuclear matrix fraction when they were maintained at 4°C but insolubilization of p48$^{v\text{-}myb}$ when exposed to 37°C (unpublished results).

Thus, hypotonic treatment of either intact cells or isolated nuclei disrupts the associations between p48$^{v\text{-}myb}$ and subnuclear structures. Our light-microscopic analysis suggests that the disruption of native nuclear structure is responsible for this phenomenon.

To determine whether there was any obvious difference in the protein composition of isotonic and hypotonic nuclei as a result of the structural alterations caused by hypotonic conditions, we analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) the polypeptide composition of both types of isolated nuclei (Fig. 4A). Among several bands which showed a quantitative decrease in hypotonic nuclei compared with isotonic nuclei, one with an M_r of 42,000 was prominently diminished. This M_r-42,000 protein, which was relatively abundant in isotonic nuclei, comigrated on SDS gels with purified actin obtained from chicken muscle fiber (Fig. 4A, lane 4) (20). Other investigators have observed a prominent nuclear actin population, which is associated with mitotic chromosomes (21), the small nuclear ribonucleoprotein complex of the peripheral lamina (9, 18), and the nuclear matrix (17). Using mono-

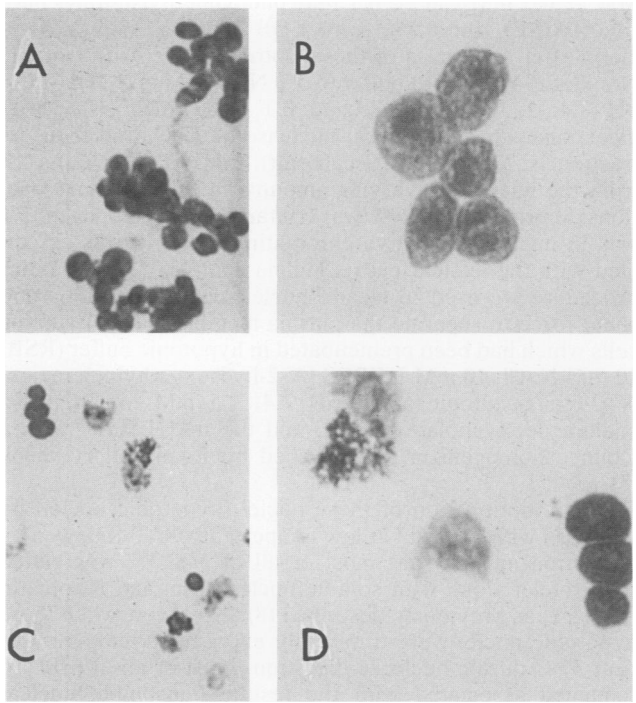

FIG. 2. Morphology of nuclei isolated from AMV-transformed myeloblasts after isotonic or hypotonic treatment. Isolated nuclei were centrifuged onto glass cover slips, fixed in absolute methanol for 6 min at −20°C, stained with May-Grunwald-Giemsa stain for 30 min, visualized by light microscopy, and photographed with Kodak Ektachrome film (ASA 400). (A) Isotonic nuclei (×100); (B) isotonic nuclei (×400); (C) hypotonic nuclei (×100); (D) hypotonic nuclei (×400).

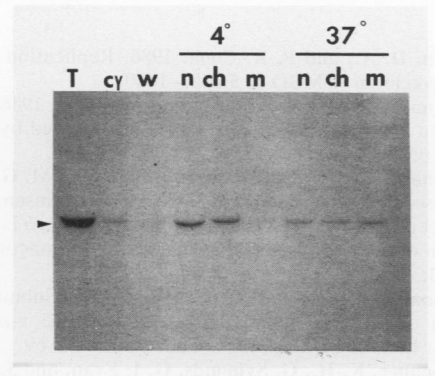

FIG. 3. Subnuclear fractionation of nuclei isolated from cells under hypotonic conditions and then incubated in isotonic buffer. BM2 myeloblasts were metabolically labeled with [^{35}S]methionine, washed in ice-cold RSB, and lysed under hypotonic conditions (8), and then purified low-salt nuclei were isolated (8). The purified low-salt nuclei were suspended in isotonic nuclear buffer without kyro EOB (1) and incubated for 30 min on ice before being fractionated into nucleoplasm, chromatin, and matrix/lamina as described in the legend to Fig. 1B. Nuclease digestion was carried out at either 4°C for 60 min or at 37°C for 15 min. Each lane represents the amount of p48$^{v\text{-}myb}$ immunoprecipitated from 10^7 cells or cell equivalents. Lanes: T, total cells; cy, cytoplasmic fraction; w, wash fraction of hypotonic nuclei; n, nucleoplasm; ch, chromatin; m, matrix/lamina.

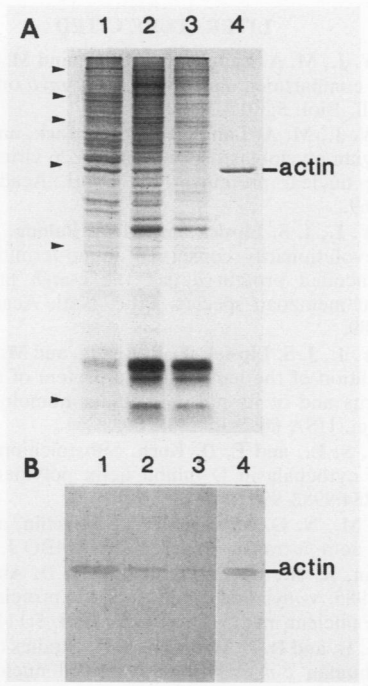

FIG. 4. (A) Polypeptide patterns of whole BM2 cells and of nuclei isolated under isotonic or hypotonic conditions. Protein samples were diluted with 1 volume of 2× electrophoresis buffer (4), boiled for 5 min at 100°C, subjected to SDS-PAGE on a 12.5% polyacrylamide gel, and then stained with Coomassie blue. Lanes: 1, lysate from 5×10^5 whole cells; 2, lysate from 10^6 isotonic nuclei; 3, lysate from 10^6 hypotonic nuclei; 4, 0.5 μg of purified actin used as a migration control. Arrows on the left represent the migration of molecular weight markers: (top to bottom) myosin (M_r 200,000), phosphorylase B (97,000), bovine serum albumin (68,000), ovalbumin (46,000), carbonic anhydrase (25,000), and soybean trypsin inhibitor (12,000). (B) Western blot of protein gels similar to those shown in panel A probed with mouse monoclonal antibody directed against chicken muscle actin (20). Immune complexes were visualized with a goat anti-mouse immunoglobulin G horseradish peroxidase conjugate (20). In lanes 1 to 3, lysates from an equal number (10^6) of cells or nuclei were analyzed. Lane 4 contained 0.5 μg of purified actin.

clonal antiactin antibodies (20) to probe a Western blot (immunoblot) of these samples, we then determined that the M_r-42,000 protein that was present in isotonic nuclei but depleted in hypotonic nuclei was indeed actin (Fig. 4B).

The findings reported here demonstrate that during the isolation of nuclei from AMV-transformed myeloblasts, the ionic strength of the extraction buffer affects the subnuclear associations of p48$^{v\text{-}myb}$ as well as of actin. After fractionation of nuclei isolated from cells under hypotonic conditions, the association of p48$^{v\text{-}myb}$ with an insoluble nuclear fraction is dependent on the temperature at which nuclease digestion occurs, as was previously reported (8, 12, 14). In contrast, the association between p48$^{v\text{-}myb}$ and an insoluble subnuclear structure generally defined as the nuclear matrix/lamina complex (1, 19) is independent of the temperature if the nuclei are isolated and treated under isotonic conditions. Also, if nuclei isolated under isotonic conditions are incubated briefly in hypotonic buffer, the nuclear associations of p48$^{v\text{-}myb}$ become temperature dependent. Thus, the apparently temperature-dependent nuclear associations of p48$^{v\text{-}myb}$ are induced by hypotonically induced changes in nuclear structure and composition. The nature of this insolubilization at temperatures near 37°C is at present unknown. We conclude that p48$^{v\text{-}myb}$ is part of a native complex which exist within the matrix/lamina (1, 3) and is preserved, at least to some extent, if nuclei are isolated and fractionated under isotonic conditions.

Various reports have demonstrated that hypotonic treatment of isolated nuclei has an adverse effect on native nuclear ultrastructure as well as on molecular interactions required for diverse nuclear functions such as DNA replication (10, 11, 16). Our finding that hypotonic treatment of cells or nuclei causes the loss of nuclear actin reinforces the concept that nuclear structural complexes are disrupted under hypotonic conditions. Several workers have noted the presence of nuclear filamentous actin structures within the chromosomal scaffold (21), the heterogeneous nuclear RNA network of the lamina (9, 18), isolated transcription complexes (6), and the filamentous matrix itself (17). Hypotonic conditions may disrupt such interactions. Interestingly, filamentous actin is converted to its globular form at low ionic strength in the presence of Mg^{2+} (5, 6), and these are precisely the conditions used in the isolation of the hypotonic nuclei described above and elsewhere (8, 12, 14). We propose that under hypotonic conditions, disruption of native nuclear complexes, possibly including nuclear actin, causes alterations in nuclear substructure and in the nuclear associations of p48$^{v\text{-}myb}$. Apparently, hypotonic conditions affect in a similar manner the intermolecular interactions of another viral transforming protein, v-*myc* (8), which is also localized within the nuclear matrix (7).

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