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Detection of a Myc-Associated Protein by Chemical Cross-Linking

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A single nuclear protein (Myc-associated protein) can be specifically cross-linked to avian Myc proteins by treatment of nuclei or cells with the reversible cross-linker dimethyl 3,3′-dithiobis-propionimidate. Myc-associated protein has a molecular weight of approximately 500,000, is not detectably phosphorylated and, in contrast to Myc, has a long apparent half-life of >3 h.

c-*myc* is one of a group of oncogenes encoding proteins found predominantly in the nucleus of the cell. This group comprises the proteins Myc, Myb, Fos, Ski, ErbA, and Jun (4, 15). Numerous studies have correlated alterations in *myc* expression with changes in regulation of cell growth and proliferation (1, 2). The nuclear localization of Myc has been widely interpreted as an indication that it participates in some growth-related nuclear process. However, despite several specific proposals, including DNA replication (14), RNA processing (13), nuclear structure (3), and transcriptional regulation (9), no biochemical function can be attributed to Myc proteins at present.

Myc binds DNA (3, 17) and RNA (V. Vogt, personal communication) in vitro, although no specific association with either nucleic acid in vivo has been established (3, 13). Nuclear Myc is phosphorylated and degraded extremely rapidly (3, 12), although little is known about the physiological role or regulation of these processes.

We have sought to identify proteins which interact or associate with Myc in the nucleus in the expectation that such information will ultimately be useful in unraveling the nature or mechanism of Myc function. A technical obstacle to identifying proteins able to form complexes with Myc is presented by the insolubility of nuclear Myc proteins in the absence of detergents or without extraction with high salt (3, 5), conditions likely to disrupt protein complexes. We reasoned that putative Myc complexes might be stabilized by chemical cross-linking prior to lysis of the cells or nuclei with detergents. Anticipating that this analysis would be facilitated by high levels of Myc expression, we chose to examine avian cell lines transformed by the avian v-*myc*-containing viruses MH2 and MC29 (7).

In initial experiments, we first metabolically labeled MH2-infected cells with [^{35}S]methionine for 60 min. Nuclei were isolated with 0.5% Nonidet P-40 in TEA buffer (50 mM triethanolamine [TEA], 10 mM NaCl, 3 mM $MgCl_2$) and then were treated with 0.5 mg of the cleavable homobifunctional imidoester cross-linker dimethyl 3,3′-dithiobis-propionimidate (DTBP, spacer arm length 1.2 nm, obtained from Pierce Chemical Co. [16]) per ml in TEA buffer for 30 min at ambient temperature (16). Whole cells were treated with 5 mg of DTBP per ml in TEA buffer. Samples were washed to remove excess cross-linker. Lysates were prepared from nuclei or cells, and the Myc proteins were collected by immunoprecipitation with anti-Myc antisera (7). The resulting immune complexes were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer and reduced with β-mercaptoethanol (4%) to cleave the cross-linker, and the eluted proteins were displayed by SDS-polyacrylamide gel electrophoresis (PAGE) (10%, wt/vol). Figure 1 shows the result of such an experiment using either whole cells or nuclei from MH2-transformed quail cells. In the absence of DTBP cross-linking, anti-Myc antisera immunoprecipitated only the p61-p63 v-Myc proteins encoded by the MH2 virus (Fig. 1, lanes 1 and 2). After DTBP cross-linking, a specific high-molecular-weight species was detected in addition to p61-p63 v-Myc (Fig. 1, lanes 4, 6, and 9). Furthermore, several proteins were nonspecifically precipitated by our antiserum specimens. These proteins, designated NS in Fig. 1, were present in immunoprecipitates with preimmune serum or where specific precipitation of Myc proteins was prevented (Fig. 1, lanes 4 and 5). DTBP treatment appeared to increase the nonspecific affinity of certain background proteins for the immune adsorbent, particularly when fixed *Staphylococcus aureus* was used for this purpose. We believe this to be due to a reactive cross-linker present on abundant proteins in the lysate. The precipitation of the NS proteins was markedly decreased by use of protein A-Sepharose (compare Fig. 1 with Fig. 2, 3, and 4), a protocol followed in all subsequent experiments. Immunoprecipitation of both p61-p63 v-Myc and the specific high-molecular-weight species can be abolished by precombining (blocking) the anti-Myc antisera with their relevant antigens (Fig. 1, lanes 5 and 7), demonstrating that immunoprecipitation of both species is specific. We designated this novel species Myc-associated protein (MYAP).

To address the possibility that MYAP might actually be a form of Myc whose electrophoretic mobility has been irreversibly altered by DTBP treatment, a portion of cross-linked lysate prepared from MH2 nuclei was treated with 10 mM dithiothreitol to cleave the cross-linker prior to the addition of anti-Myc antisera. Cleavage of the cross-linker prior to incubation with antibody eliminates immunoprecipitation of MYAP, while recovery of p61-p63 v-Myc is unchanged (Fig. 2, lanes 2 and 4). This demonstrates that anti-Myc antibodies do not react with MYAP itself, strongly suggesting that MYAP is not immunologically related to Myc but instead is coimmunoprecipitated by virtue of its physical proximity to Myc.

The specificity of association of MYAP with Myc was investigated by comparing MH2 cells, which express v-Myc and Gag proteins predominantly as distinct species, with Q8 nonproducer cells, which express v-Myc as a 110-kilodalton Gag-Myc fusion protein. Nuclei from Q8 or MH2 cells were cross-linked with DTBP and immunoprecipitated with both anti-Myc and anti-Gag antisera (Fig. 3). In Q8 lysate, MYAP

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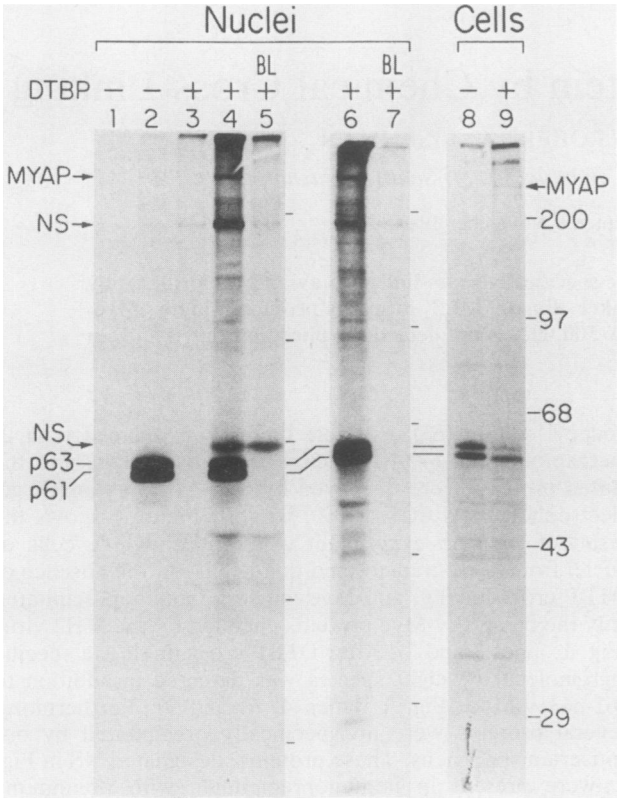

FIG. 1. Analysis of anti-Myc immunoprecipitates from DTBP cross-linked nuclei and whole cells. Nuclei and cells were prepared as described in the text. Anti-Δ22-Myc is a broadly reactive antiserum raised against bacterially expressed human c-Myc protein (10); anti-v-Myc 12C is an avian v-Myc- and c-Myc-restricted anti-C-terminal peptide antiserum (7). BL, Blocked, i.e., antiserum precombined with peptide immunogen; NS, nonspecific protein band. +, DTBP added; −, no DTBP added. Numbers at right indicate molecular weights. Lanes: 1, preimmune antiserum; 2, anti-Δ22-Myc; 3, preimmune serum; 4, anti-Δ22-Myc; 5, anti-Δ22-Myc precombined with bacterially expressed human c-Myc protein; 6, anti-v-Myc 12C avian C-terminal peptide antiserum; 7, anti-v-Myc 12C precombined with C-terminal peptide; 8, anti-v-Myc 12C; 9, anti-v-Myc 12C.

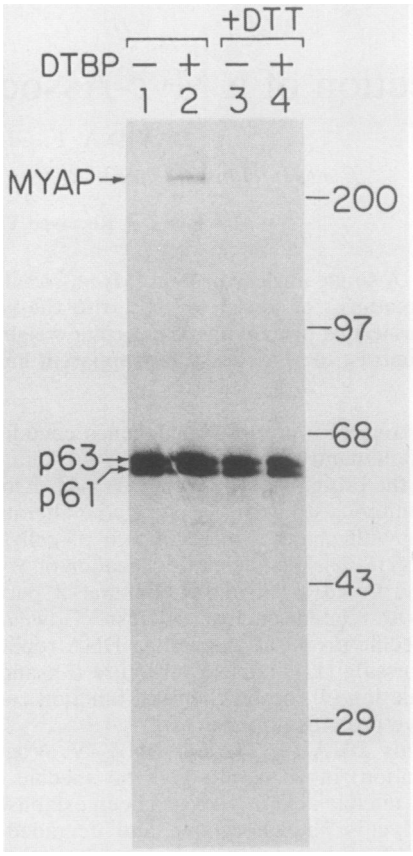

FIG. 2. Dithiothreitol treatment of cross-linked Myc prior to immunoprecipitation. MH2-infected cells were labeled with [^{35}S]methionine for 60 min, and nuclei were treated with DTBP as described in the text. One half of the lysate was treated with 10 mM dithiothreitol (DTT) before precipitation with anti-v-Myc 12C followed by elution in sample buffer, reduction, and analysis by 10% (wt/vol) SDS-PAGE. Numbers at right indicate molecular weights.

was immunoprecipitated by both anti-Myc and anti-Gag antisera (Fig. 3, lanes 1 and 3) but only by anti-Myc antiserum in MH2 lysate (Fig. 3, lanes 4 and 6), even though the amount of Gag-related proteins (predominantly Pr76^{gag}) collected by immunoprecipitation was quantitatively similar to that of p61-p63 v-Myc. Thus, MYAP can be cross-linked to P110$^{gag\text{-}myc}$ but not to the *gag*-encoded proteins themselves. Finally, MYAP is not coimmunoprecipitated, after DTBP cross-linking, with lamin A, an abundant cellular protein found in the nucleus or with *v*-Myb proteins (data not shown). Taken together, these results suggest that DTBP acts to stabilize v-Myc and MYAP in a specific spatial relationship which would otherwise be disrupted during lysis of nuclei and whole cells.

Although indirect, coimmunoprecipitation with anti-Myc antiserum has allowed us to establish some properties of MYAP. The conclusion that MYAP is a protein is based on the observations that it is metabolically labeled with [^{35}S]methionine, is sensitive to proteinase K, and can be detected by silver staining (data not shown). MYAP was unaffected by DNase I digestion of immunocomplexes under conditions in which 1 μg of exogenously added bacteriophage lambda DNA was completely digested. Accurate measurement of molecular weight was hampered by migration of MYAP in the nonlinear portion of even low-percentage SDS-PAGE gels (Fig. 3 and 4); however, by comparison with bovine thyroglobulin (molecular weight, 400,000), we estimate MYAP to have a molecular weight of approximately 500,000. Attempts to detect MYAP by using metabolic labeling with $^{32}PO_4$ in conditions under which Myc and other phosphoproteins were labeled were unsuccessful, suggesting that MYAP may not be a phosphoprotein (data not shown).

Since one of the most striking features of the *myc*-encoded proteins is their rapid turnover rate (4, 12), it was of interest to determine the kinetics of the relationship of MYAP to Myc during a pulse-chase experiment. MH2 cells were pulse-labeled for 15 min with [^{35}S]methionine and subsequently chased in unlabeled medium containing excess nonradioactive methionine. At various times after labeling, nuclei were prepared, cross-linked with DTBP, and immunoprecipitated with anti-Myc antiserum. p61-p63 v-Myc proteins were metabolized extremely rapidly with a half-life of around 20 min (Fig. 4, lanes 1 through 5). In marked contrast, radioactive MYAP became coimmunoprecipitated only after a lag phase of approximately 30 min (Fig. 4, lanes 1 and 2) and continued to be precipitated throughout the

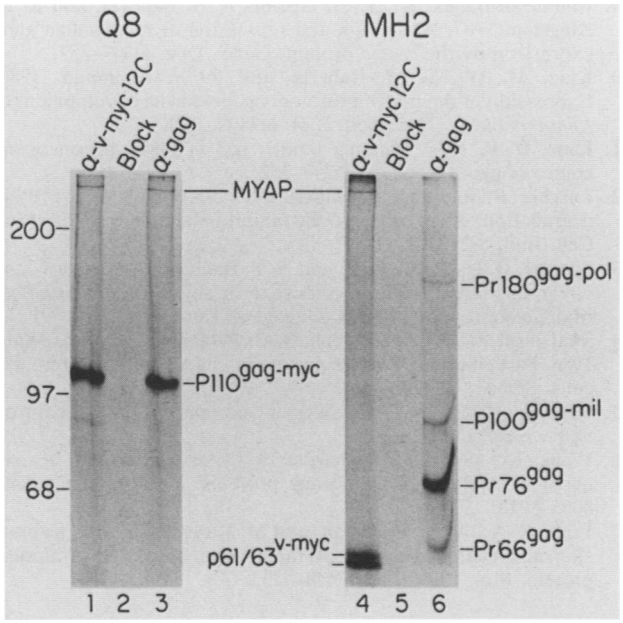

FIG. 3. Immunoprecipitations of DTBP cross-linked *myc*-, *gag-myc*-, and *gag*-encoded proteins. MH2-infected and MC29-infected (Q8) cells were labeled with [^{35}S]methionine for 60 min, and nuclei were treated with DTBP as described in the text. Lanes: 1, anti-v-Myc 12C; 2, anti-v-Myc 12C precombined with C-terminal peptide (Block); 3, 5202 anti-Gag antiserum; 4, anti-v-Myc 12C; 5, anti-v-Myc 12C precombined with C-terminal peptide (Block); 6, 5202 anti-Gag antiserum. Immunocomplexes were eluted, reduced, and analyzed by 5% (wt/vol) SDS-PAGE.

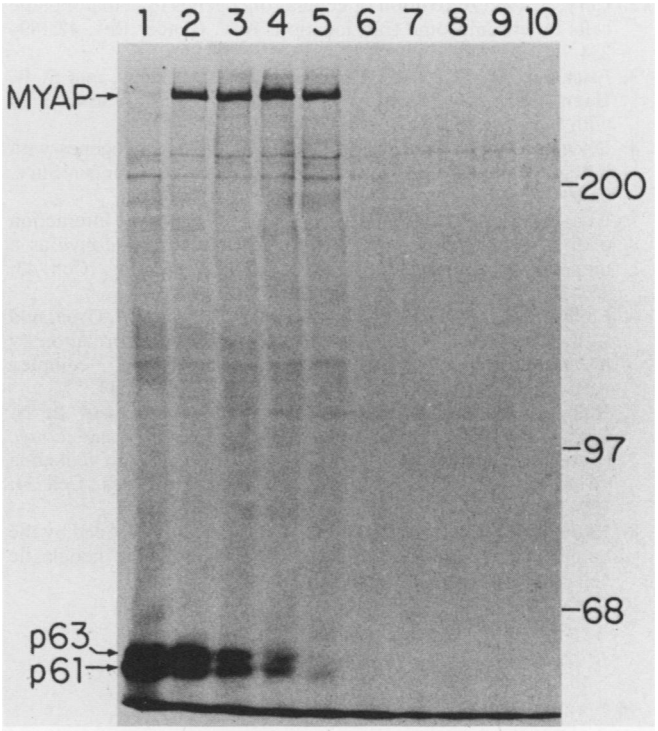

FIG. 4. Kinetics of Myc-MYAP cross-linking. MH2-infected cells were labeled with [^{35}S]methionine for 15 min and then were transferred to medium containing excess nonradioactive methionine for 30, 60, 90, or 180 min. Nuclei were isolated and treated with DTBP as described in the text. In lanes 1 through 5, all samples were precipitated with anti-v-Myc 12C. Lanes: 1, pulse; 2, 30-min chase; 3, 60-min chase; 4, 90-min chase; 5, 180-min chase. Lanes 6 through 10 represent same pulse or chase periods as in lanes 1 through 5 except anti-v-Myc 12C antiserum was precombined with avian C-terminal peptide. Samples were reduced and analyzed by 5% (wt/vol) SDS-PAGE.

chase period (3 h). We presume that MYAP remains immunoprecipitable either by virtue of its association with unlabeled Myc, newly synthesized during the chase period, or with a small subpopulation of stable Myc. The delay in association of labeled MYAP with v-Myc may represent the time period necessary for the synthesis or transport to the nucleus of such a large molecule or alternatively may reflect some rate-limiting step in physical association. The conclusion that MYAP has a longer apparent half-life than Myc is tentative, subject to the assumption that the immunoprecipitated MYAP is representative of the total nonprecipitated MYAP.

Several lines of evidence presented above suggest that MYAP may specifically interact with v-Myc in the nucleus. MYAP can also be coimmunoprecipitated with c-Myc proteins in the chicken bursal lymphoma cell line BK3A (data not shown), which expresses high levels of c-Myc (7). Normal chicken and quail cells express such low levels of c-Myc proteins that association with MYAP cannot be determined by using these procedures (D. A. F. Gillespie, unpublished results).

While our results imply that molecules of MYAP and Myc are closely associated (within the 1.2-nm spacer arm length of DTBP), our data does not address the important question of whether the association represents a functional interaction. It is possible, for example, that cross-linking of MYAP and Myc may be a consequence of Myc overexpression, with excess Myc being aberrantly sequestered in a nuclear compartment in proximity to MYAP. Nonetheless, although MYAP is an anonymous species, its unusual physical properties suggest that further characterization of MYAP itself and of the Myc-MYAP interaction may be informative in terms of Myc function. Particularly intriguing is the close association of two proteins with such apparently different rates of degradation. It will be of considerable interest to determine the stability of the subset of Myc proteins which associate with MYAP. If the components of the complex are present in equimolar amounts, then the associated Myc may well be a very small fraction of the total Myc. One possibility is that association with MYAP might lead to stabilization of a small subset of Myc in a manner analogous to that of the stabilization of p53 by association with simian virus 40 large T antigen and HSP 70 (6, 11). Alternatively, Myc in Myc-MYAP complexes may be rapidly degraded, suggesting a possible role for MYAP in Myc protein degradation. To answer these specific questions and to characterize and hopefully identify MYAP, it will be important to generate anti-MYAP antisera.

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