MOLECULAR AND CELLULAR BIOLOGY, Jan. 1986, p. 62–69
0270-7306/86/010062-08$02.00/0

Vol. 6, No. 1

Subnuclear Localization of Proteins Encoded by the Oncogene v-*myb* and Its Cellular Homolog c-*myb*

KARL-HEINZ KLEMPNAUER* AND ALBRECHT E. SIPPEL

Zentrum für Molekulare Biologie der Universität Heidelberg, Im Neuenheimer Feld 282, D-69 Heidelberg, Federal Republic of Germany

Received 9 July 1985/Accepted 9 October 1985

The retroviral transforming gene v-*myb* encodes a 45,000-M_r nuclear transforming protein ($p45^{v\text{-}myb}$). $p45^{v\text{-}myb}$ is a truncated and mutated version of a 75,000-M_r protein encoded by the chicken c-*myb* gene ($p75^{c\text{-}myb}$). Like its viral counterpart, $p75^{c\text{-}myb}$ is located in the cell nucleus. As a first step in identifying nuclear targets involved in cellular transformation by v-*myb* and in c-*myb* function, we determined the subnuclear locations of $p45^{v\text{-}myb}$ and $p75^{c\text{-}myb}$. Approximately 80 to 90% of the total $p45^{v\text{-}myb}$ and $p75^{c\text{-}myb}$ present in nuclei was released from nuclei at low salt concentrations, exhibited DNA-binding activity, and was attached to nucleoprotein particles when released from the nuclei after digestion with nuclease. A minor portion of approximately 10 to 20% of the total $p45^{v\text{-}myb}$ and $p75^{c\text{-}myb}$ remained tightly associated with the nuclei even in the presence of 2 M NaCl. These observations suggest that both proteins are associated with two nuclear substructures tentatively identified as the chromatin and the nuclear matrix. The function of *myb* proteins may therefore depend on interactions with several nuclear targets.

Avian myeloblastosis virus (AMV) is an acutely oncogenic avian retrovirus that causes myeloblastic leukemia in chickens and transforms cells of the myelomonocytic lineage in vitro (31). The oncogenicity of AMV is attributed to a transforming gene, termed v-*myb*, that resides at the 3′ end of the viral genome (17, 24, 26, 40). The product encoded by v-*myb* has been identified as a 45,000-M_r protein, $p45^{v\text{-}myb}$, that is located primarily in the nucleus of myeloblasts transformed by AMV (10, 27, 28). The viral *myb* gene is a partial copy of a normal cellular gene referred to as c-*myb* (22, 26, 40). c-*myb* has been conserved during evolution and is expressed primarily in immature hematopoietic cells (23, 39). In chickens, c-*myb* encodes a 75,000-M_r protein, $p75^{c\text{-}myb}$ (27). Like its viral counterpart, $p75^{c\text{-}myb}$ also is a nuclear protein (28).

Retroviral transforming genes are commonly classified into families of related oncogenes, based on the properties of their protein products. Examples of such families are the classes of oncogenes that encode GTP-binding and -hydrolyzing molecules, protein kinases, and nuclear proteins (8, 25). Because of their close association with the cell nucleus, *myb* proteins are members of the group of nuclear transforming proteins. In addition to v-*myb*, this family of transforming genes includes the v-*myc* gene of avian myelocytomatosis virus MC29 and of related viruses and the v-*fos* gene of the FBR and FBJ strains of mouse osteosarcoma viruses (1, 3, 15, 16). The existence of families of functionally related transforming proteins supports the concept that proliferation of cells is controlled by a number of distinct regulatory events that appear to take place at various locales within the cell. However, all regulatory mechanisms that are involved in controlling cell proliferation must somehow be linked to the cellular genome to regulate its replication. It is conceivable that retroviral oncogenes encoding nuclear proteins participate in or interfere with regulatory processes that directly involve the DNA. The analysis of the function of nuclear transforming proteins may therefore provide insight into the molecular details of control of cell proliferation on the level of the genome.

One important step in the analysis of oncogene function is the identification and characterization of cellular targets that interact with the transforming proteins. To identify targets of the viral and cellular *myb* proteins we determined, as a first step, the subnuclear location of $p45^{v\text{-}myb}$ and $p75^{c\text{-}myb}$. Our results suggest that both proteins associate with two nuclear substructures tentatively identified as the chromatin and the nuclear matrix. The function of *myb* proteins may therefore depend on interactions with several nuclear targets.

MATERIALS AND METHODS

Cells. Cells of the BM2 line of AMV-transformed chicken myeloblasts (32) were obtained from C. Moscovici. Chicken erythroblasts transformed by the ES-4 strain of avian erythroblastosis virus (AEV) were provided by T. Graf. Myeloblasts were propagated in RPMI 1640 medium supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, and 5% chicken serum. Erythroblasts were cultivated in RPMI 1640 medium supplemented with 8% fetal calf serum and 2% chicken serum.

Antisera. Immunoprecipitations were performed with polyclonal rabbit antiserum raised against bacterially expressed v-*myb* protein as described previously (27). Immunoblotting was performed with culture supernatant of the *myb*2-37 hybridoma line prepared as described previously (19).

Labeling of cells. Cells to be radioactively labeled with [^{35}S]methionine were pelleted from growth medium and washed twice with prewarmed serum-free Eagle minimal essential medium lacking methionine. Cells (5×10^6 to 10^7/ml) were then incubated in methionine-free minimal essential medium without serum for 30 min. Radioactive labeling was started by adding [^{35}S]methionine (specific activity, >600 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) to a final concentration of 250 μCi/ml. Labeling was continued for 1 h and was stopped by pelleting the cells and washing them twice with ice-cold phosphate-buffered saline.

* Corresponding author.

Preparation and fractionation of nuclei. To prepare nuclei, cells were pelleted and washed twice in ice-cold hypotonic buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0); 5 mM KCl, and 2 mM $MgCl_2$. They were then incubated in hypotonic buffer for 5 to 10 min on ice and disrupted in a Dounce homogenizer with a tightly fitting pestle until virtually all cells were broken (usually 25 strokes). The extent of breakage of cells was monitored microscopically. Nuclei were then separated from the cytoplasmic fraction by sedimentation at 1,000 × *g* for 5 min and washed twice with ice-cold hypotonic buffer. The nuclei were then suspended in ice-cold hypotonic buffer and kept on ice. All fractionation experiments were performed with freshly prepared nuclei at a concentration of 10^8 nuclei per ml. In salt extraction experiments a solution containing 5 M NaCl was used to adjust the suspension of nuclei to the desired salt concentration. When extraction in the presence of 2 M NaCl was required, the nuclei were suspended directly in hypotonic buffer supplemented with 2 M NaCl. In nuclease digestion experiments appropriate amounts of a solution containing 2 mg of DNaseI per ml were added to the nuclei to obtain the desired concentration of nuclease. All extractions were performed for 10 min at 0°C. Nuclease treatment was terminated by adding EDTA to a final concentration of 5 mM followed by an additional incubation for 10 min on ice. Nuclear supernatants and residual nuclei were separated by centrifugation. Fractions to be analyzed by immunoblotting were mixed with an equal volume of twofold-concentrated sample buffer (29) and boiled before loading onto gels. Fractions to be analyzed by agarose gel electrophoresis were adjusted to 0.5% sodium dodecyl sulfate (SDS), incubated with 100 μg of proteinase K per ml at 37°C for 30 min, and electrophoresed in 1.2% agarose gels. DNA was visualized by staining with ethidium bromide. Fractions obtained by sequential extraction of nuclei were precipitated with 5 volumes of acetone for 12 h at −20°C. Fractions containing 2 M NaCl were diluted fivefold with water before acetone precipitation. Precipitated proteins to be analyzed by immunoprecipitation were dissolved by boiling in 1% SDS. Samples were then diluted 10-fold with buffer containing 10 mM Tris hydrochloride (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40 before addition of antiserum. Precipitated proteins to be analyzed by immunoblotting were dissolved by boiling directly in sample buffer (29).

Velocity gradient sedimentation. Samples containing nucleoprotein complexes isolated from nuclease-treated nuclei were sedimented through linear 5 to 30% sucrose gradients made in 10 mM HEPES (pH 8.0)–1 mM EDTA (low salt) or 10 mM HEPES (pH 8.0)–1 mM EDTA–500 mM NaCl (high salt). Centrifugation was performed for 2.5 h at 4°C and 45,000 rpm in an SW60 rotor. Samples of each fraction were mixed with an equal volume of twofold-concentrated sample buffer (29), boiled, and analyzed by immunoblotting. Alternatively samples of gradient fractions were adjusted to 0.5% SDS, incubated with 100 μg of proteinase K per ml at 37°C for 30 min, and then analyzed by electrophoresis in 1.2% agarose gels.

Immunoprecipitation. Immunoprecipitation of *myb* proteins with polyclonal *myb*-specific rabbit serum has been described previously (27).

Immunoblotting. Protein samples to be analyzed by immunoblotting were fractionated in 10% SDS–polyacrylamide gels (29). Gels were blotted onto nitrocellulose (BA85; Schleicher & Schuell, Inc., Keene, N.H.) as described previously (43). All further manipulations were performed at room temperature. The blots were rinsed briefly with TBS (144 mM NaCl, 10 mM Tris hydrochloride, pH 7.8) and then incubated for 2 h in TBS containing 1% bovine serum albumin. Subsequently, the blots were incubated in culture supernatant of the *myb*2-37 hybridoma line (19) supplemented with 1% bovine serum albumin. The blots were then washed three times (15 min each wash) with buffer containing 10 mM Tris hydrochloride (pH 7.8), 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, and 0.1% SDS and then incubated in swine anti-mouse immunoglobulin G coupled to horseradish peroxidase (Dako) at a dilution of 1:500 in TBS containing 1% bovine serum albumin. The incubation was continued for 1 h and terminated by three washes (10 min per wash) with TBS supplemented with 0.5% Nonidet P-40. The blots were then rinsed briefly with TBS and stained with a solution containing 25 mg of diaminobenzidine, 30 μl of H_2O_2, and 1.5 ml of 1% $CoCl_2$ per 50 ml of TBS. Staining was stopped by washing the blots with water and drying them.

DNA-cellulose chromatography. All manipulations were performed on ice or at 4°C. Nuclei were prepared from radioactively labeled cells as described above. Proteins were extracted by incubating the nuclei at a concentration of 10^8 nuclei per ml in buffer containing 10 mM HEPES (pH 8.0), 1 mM EDTA, and 0.4 M NaCl for 30 min on ice. The nuclei were then pelleted by centrifugation at 10,000 × *g* for 30 min. The supernatant was diluted 10-fold with ice-cold buffer containing 10 mM HEPES (pH 8.0) and 1 mM EDTA and clarified again (10,000 × *g*, 20 min). The supernatant was then loaded at a flow rate of 4 ml/h onto a column of native calf thymus DNA-cellulose (bed volume approximately 1 ml; Sigma Chemical Co., St. Louis, Mo.) equilibrated with buffer containing 10 mM HEPES (pH 8.0), 1 mM EDTA, and 40 mM NaCl. The column was then washed with approximately 10 bed volumes of equilibration buffer at a flow rate of 4 ml/h to remove any unbound protein. Bound protein was then eluted with a linear gradient of 50 mM to 1 M NaCl in buffer containing 10 mM HEPES (pH 8.0) and 1 mM EDTA at a flow rate of 2 ml/h. The total volume of the gradient was 10 ml. Samples of the loaded material and the flowthrough, wash, and gradient fractions were analyzed by immunoprecipitation.

RESULTS

Release of $p45^{v\text{-}myb}$ from nuclease-treated nuclei of AMV-transformed myeloblasts. To analyze the role of DNA in nuclear association of the viral *myb* protein, we incubated nuclei prepared from AMV-transformed myeloblasts in the presence of increasing amounts of DNaseI. The release of $p45^{v\text{-}myb}$ from the nuclei was monitored by immunoblotting with the *myb*-specific monoclonal antibody *myb*2-37 (19). In addition to recognizing $p45^{v\text{-}myb}$, the *myb*2-37 antibody reacts with another nuclear protein with an apparent molecular weight of 37,000 (referred to as p37) present in AMV-transformed myeloblasts. p37 is not a proteolytic cleavage product of $p45^{v\text{-}myb}$ since the smaller protein is present in a large variety of avian and mammalian cell lines not expressing $p45^{v\text{-}myb}$ (19). Although p37 has not been characterized further and its relationship to other nuclear proteins is not known, we use it in some experiments as an internal control. Figure 1A illustrates that whereas $p45^{v\text{-}myb}$ was completely retained by the nuclei after an incubation in the absence of nuclease, even very mild nuclease digestion of cellular DNA released approximately 80 to 90% of the total $p45^{v\text{-}myb}$ from the nuclei. Under these conditions most of the DNA was still present as fragments that were, on the average, several

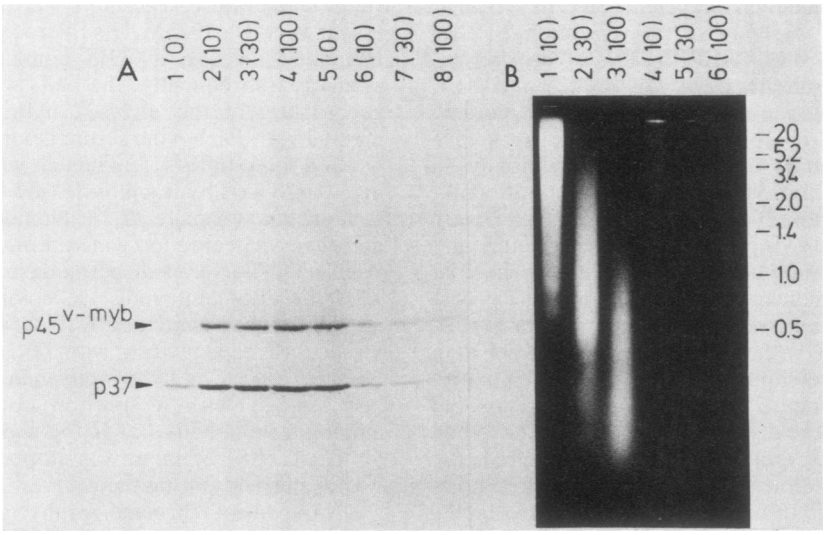

FIG. 1. Release of $p45^{v\text{-}myb}$ from nuclei of AMV-transformed myeloblasts by DNase I. Nuclei (5×10^6) isolated from BM2 cells were digested with DNaseI at concentrations ranging from 0 to 100 μg/ml for 10 min at 0°C. Numbers appearing in parentheses above the lanes indicate the concentrations of DNaseI used. (A) Nuclear supernatants (lanes 1 to 4) and residual nuclei (lanes 5 to 8) were analyzed by immunoblotting as described in Materials and Methods. The positions of $p45^{v\text{-}myb}$ and p37 are marked. (B) DNA fragments released from nuclei (lanes 1 to 3) or retained by nuclei (lanes 4 to 6) after incubation with DNaseI were analyzed by agarose gel electrophoresis. The numbers at the side refer to the sizes (in kilobase pairs) and positions of DNA size markers.

kilobase pairs in length (Fig. 1B). Approximately 10 to 20% of the total $p45^{v\text{-}myb}$ remained associated with the nuclei during nuclease treatment. The size of this fraction appeared not to depend on the extent of nucleolytic cleavage of the cellular DNA. The experiment shown in Fig. 1 was not designed to achieve complete digestion of cellular DNA. Quantitation of the amount of DNA released and retained by the nuclei indicated that about 5% of the total DNA remained associated with the nuclei after nuclease treatment.

At physiological salt concentrations DNA released from nuclease-treated nuclei is contained in nucleoprotein complexes that exhibit characteristic features of chromatin structure such as nucleosomal organization or histone H1-dependent higher levels of chromatin organization (see reference 45 for a recent example). To determine whether $p45^{v\text{-}myb}$ is attached to nucleoprotein fragments, we sedimented the material released from nuclease-treated nuclei through sucrose gradients (Fig. 2). The distribution of $p45^{v\text{-}myb}$ throughout the gradient, as determined by immunoblotting with the *myb*2-37 antibody, followed a heterogeneous pattern similar to that observed for DNA or histones (panels A to C). In the presence of 0.5 M NaCl all the $p45^{v\text{-}myb}$ sedimented in the upper fractions of the gradient, suggesting that the protein was released from fast-sedimenting particles (panel D). At the same time the sedimentation profile of DNA or histones was shifted only slightly (approximately by one fraction) toward the top of the gradient (data not shown). To further demonstrate that $p45^{v\text{-}myb}$ is bound to chromatin fragments, we sedimented material that had been released from nuclei by incubation at different concentrations of nuclease through sucrose gradients (Fig. 3). We found that, as the extent of nucleolytic cleavage of cellular DNA increased, particles containing DNA and histones as well as $p45^{v\text{-}myb}$ sedimented more slowly. We also observed that the broad peak of viral *myb* protein was shifted slightly toward the top of the gradient relative to the distribution of histones or DNA fragments (compare, for example, panels C and D of Fig. 3).

Release of $p45^{v\text{-}myb}$ from salt-treated nuclei of AMV-transformed myeloblasts. The attachment of $p45^{v\text{-}myb}$ to nucleoprotein fragments and the release of the protein from these fragments by salt suggests a salt-labile association of the transforming protein with cellular chromatin. We therefore investigated whether $p45^{v\text{-}myb}$ can be released from intact nuclei by salt treatment. The experiment illustrated in Fig. 4 shows that whereas very little $p45^{v\text{-}myb}$ was released from nuclei during an incubation in buffer to which no salt had been added, a concentration of 300 mM NaCl was effective in extracting 80 to 90% of the total $p45^{v\text{-}myb}$ from the nuclei. Increasing the salt concentration to 400 mM NaCl did not release more $p45^{v\text{-}myb}$. A small fraction of approximately 10 to 20% of the total $p45^{v\text{-}myb}$ was retained by the nuclei. We noted that p37 was extracted quantitatively under these conditions.

To investigate the relationship between the fractions of $p45^{v\text{-}myb}$ that were released from nuclei by either salt treatment or nucleolytic digestion of DNA and the fraction that remained stably associated with the nuclei, we extracted nuclei from AMV-transformed myeloblasts sequentially with hypotonic buffer, 0.4 M NaCl, DNaseI, and 2 M NaCl (Fig. 5A). This experiment demonstrated that after an initial extraction of $p45^{v\text{-}myb}$ by 0.4 M NaCl further treatment of nuclei with DNaseI did not result in the release of any additional $p45^{v\text{-}myb}$, whereas the residual fraction of viral *myb* protein remaining associated with the nuclei was not decreased relative to the experiment shown in Fig. 4. In another series of extractions, we reversed the order of the 0.4 M NaCl and nuclease extraction steps (Fig. 5B). In this case, nuclease treatment led to release of most of the $p45^{v\text{-}myb}$ from the nuclei, whereas further incubation in the presence of 0.4 M NaCl solubilized little additional $p45^{v\text{-}myb}$ and apparently did not affect the size of the residual fraction of transforming protein that remained associated with the nucleus. We conclude that a major portion (80 to 90%) of $p45^{v\text{-}myb}$ is released from nuclei by treatment with either 0.4 M NaCl or after nuclease digestion. A smaller fraction (10 to

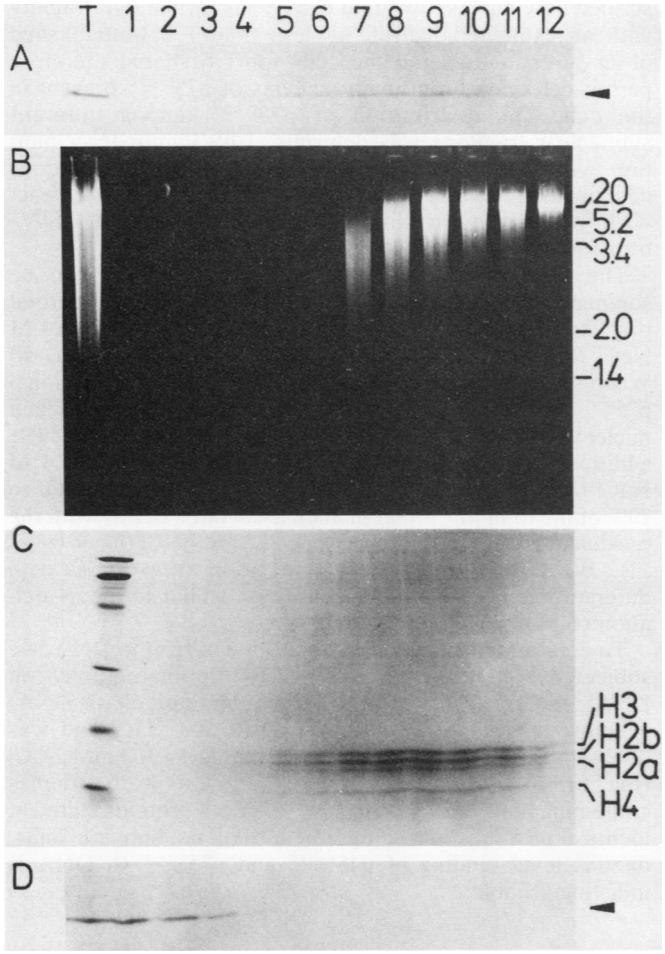

FIG. 2. Velocity gradient sedimentation of nucleoprotein complexes released from nuclei of AMV-transformed myeloblasts by nuclease. Nuclei (4×10^7) from BM2 cells were digested in the presence of 10 μg of DNase I per ml. The material released after treatment of the nuclei with EDTA was divided into two samples of 200 μl each. The salt concentration in one sample was adjusted to 0.5 M NaCl. One-third of each sample was analyzed directly (lane T), and the remaining material was subjected to velocity gradient sedimentation as described in Materials and Methods. Numbered lanes refer to individual gradient fractions; sedimentation was from left to right (top fraction is lane 1). A shows an immunoblot analysis of the low-salt gradient; D shows an immunoblot analysis of the gradient containing 0.5 M NaCl. Only the relevant portions of the immunoblots are shown. The arrows indicate the position of $p45^{v\text{-}myb}$. B shows the size distribution of DNA fragments throughout the low-salt gradient as determined by agarose gel electrophoresis. The numbers at the side indicate the sizes (in kilobase pairs) and positions of DNA size markers. C shows the distribution of proteins throughtout the low-salt gradient as determined by electrophoresis in a 14% SDS–polyacrylamide gel. The positions of the core histones are marked. Lane T of panel C shows protein size markers with the following molecular weights ($\times 10^3$; from top): 93, 66, 43, 31, 21.5, 14.4.

20%) of $p45^{v\text{-}myb}$ is not extracted by these procedures and remains in the nucleus. The association of this fraction of $p45^{v\text{-}myb}$ with the nucleus is very tight as it is maintained even in the presence of 2 M NaCl. This interaction, however, is sensitive to treatment with detergent. Incubation of nuclei in the presence of 0.1% SDS resulted in complete solubilization of $p45^{v\text{-}myb}$ (Fig. 5C).

Binding of $p45^{v\text{-}myb}$ to DNA. To investigate whether the interaction of $p45^{v\text{-}myb}$ with chromatin might be the result of binding of the viral *myb* protein to DNA, we subjected the salt-extractable fraction of $p45^{v\text{-}myb}$ to chromatography on DNA-cellulose (Fig. 6). Virtually all the salt-extracted $p45^{v\text{-}myb}$ bound to DNA-cellulose at a salt concentration of 40 mM NaCl and was released from the column at an NaCl concentration of approximately 300 mM. Binding of $p45^{v\text{-}myb}$ to the column could be blocked by adding unbound competitor DNA to the salt-extracted protein. This suggests that $p45^{v\text{-}myb}$ binds to the DNA attached to the cellulose matrix and not to the matrix itself (data not shown).

Subnuclear localization and DNA binding of $p75^{c\text{-}myb}$. To determine whether the protein encoded by c-*myb*, $p75^{c\text{-}myb}$, shows a similar association with different nuclear substructures as $p45^{v\text{-}myb}$, we repeated some of the experiments described above using nuclei prepared from AEV-trans-

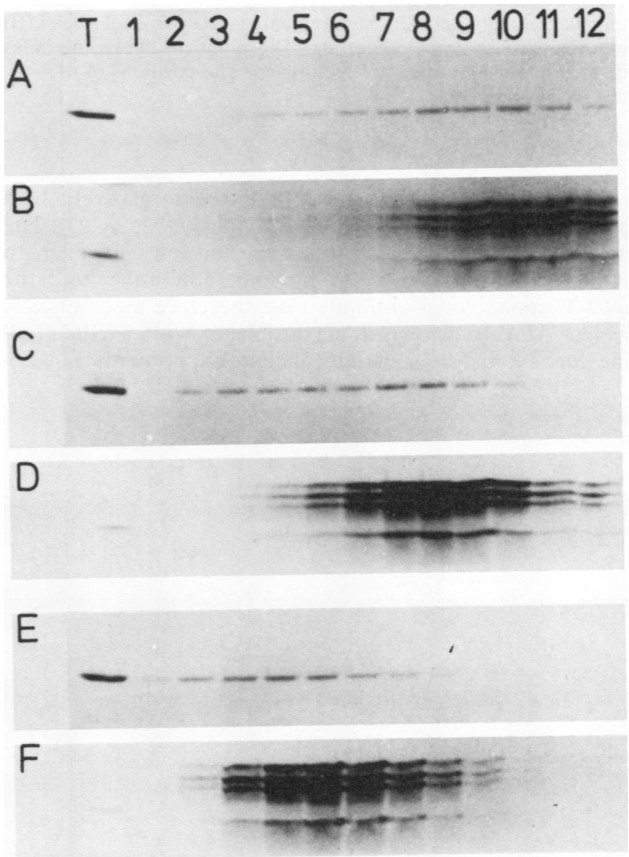

FIG. 3. Sedimentation of nucleoprotein complexes released by different concentrations of nuclease. Approximately 4×10^7 nuclei from BM2 cells were digested by 10 (A and B), 30 (C and D), or 100 (E and F) μg of DNase I per ml. One-third of the material released after treatment of the nuclei with EDTA was analyzed directly (lane T of panels A, C, and E). The remaining material was subjected to velocity gradient sedimentation in low salt. The numbering of lanes and the direction of sedimentation are the same as in Fig. 2. Samples of the resulting gradient fractions were electrophoresed in 10% (panels A, C, and E) or 14% (panels B, D, and F) SDS–polyacrylamide gels and analyzed by immunoblotting (panels A, C, and E) or by staining (panels B, D, and F). Only the relevant portions of the resulting blots and gels (encompassing $p45^{v\text{-}myb}$ and the core histones, respectively) are shown. In panels B, D, and F, lane T shows lysozyme used as a size marker (14.4 kilodaltons).

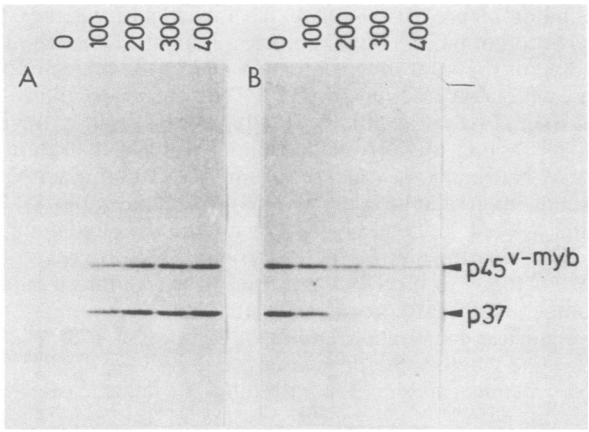

FIG. 4. Salt extraction of $p45^{v\text{-}myb}$ from nuclei of AMV-transformed myeloblasts. Nuclei (3×10^6) isolated from BM2 cells were incubated in the presence of NaCl at concentrations ranging from 0 to 400 mM. Incubations were performed for 10 min at 0°C. Samples containing nuclear supernatants (A) or residual nuclei (B) were analyzed by immunoblotting. The numbers above the lanes indicate the concentrations of NaCl used. The positions of $p45^{v\text{-}myb}$ and p37 are indicated.

formed erythroblasts. These cells express relatively high levels of c-*myb* protein as shown previously (22, 23, 27). The intracellular levels of this protein are too low, however, to permit detection of $p75^{c\text{-}myb}$ by immunoblotting with the *myb*2-37 monoclonal antibody. We therefore metabolically labeled AEV-transformed erythroblasts with [^{35}S]methionine for 1 h before isolating nuclei. Experiments not described here have shown that $p75^{c\text{-}myb}$ is turned over rapidly with an estimated half-life of less than 1 h (unpublished observations). Labeling the cells for 1 h should therefore permit detection of all major species of $p75^{c\text{-}myb}$ present in the cell. The distribution of $p75^{c\text{-}myb}$ among different subnuclear fractions was determined by immunoprecipitation with polyclonal *myb*-specific antiserum prepared as described previously (27). This antiserum does not react with p37 although this protein is present in AEV-transformed erythroblasts (19).

The results of sequential extractions (Fig. 7) can be summarized as follows. (i) About 80 to 90% of the total $p75^{c\text{-}myb}$ was released from nuclei in the presence of 0.4 M NaCl (lane 3) or after digestion with DNaseI (lane 8). (ii) When nuclei were extracted with 0.4 M NaCl, no additional $p75^{c\text{-}myb}$ was released by nuclease treatment (lane 4). When nuclei were first incubated in the presence of nuclease, little additional $p75^{c\text{-}myb}$ was released in the presence of 0.4 M NaCl (lane 9). (iii) A small fraction of approximately 10 to 20% of the total $p75^{c\text{-}myb}$ remained associated tightly with the residual nuclei even in the presence of 2 M NaCl (lanes 6 and 11). We conclude that the association of $p75^{c\text{-}myb}$ with different nuclear substructures is very similar to the association of $p45^{v\text{-}myb}$ with the nucleus.

To determine whether $p75^{c\text{-}myb}$ is able to bind to DNA, we subjected salt-extracted $p75^{c\text{-}myb}$ to chromatography on DNA-cellulose. $p75^{c\text{-}myb}$ was bound quantitatively to DNA-cellulose at a salt concentration of 40 mM NaCl and was released by a concentration of approximately 300 mM NaCl (Fig. 8). In cochromatography experiments with samples containing both $p45^{v\text{-}myb}$ and $p75^{c\text{-}myb}$, both proteins eluted at identical positions in a salt gradient (data not shown). Thus, on this level binding of $p45^{v\text{-}myb}$ and $p75^{c\text{-}myb}$ to DNA is indistinguishable.

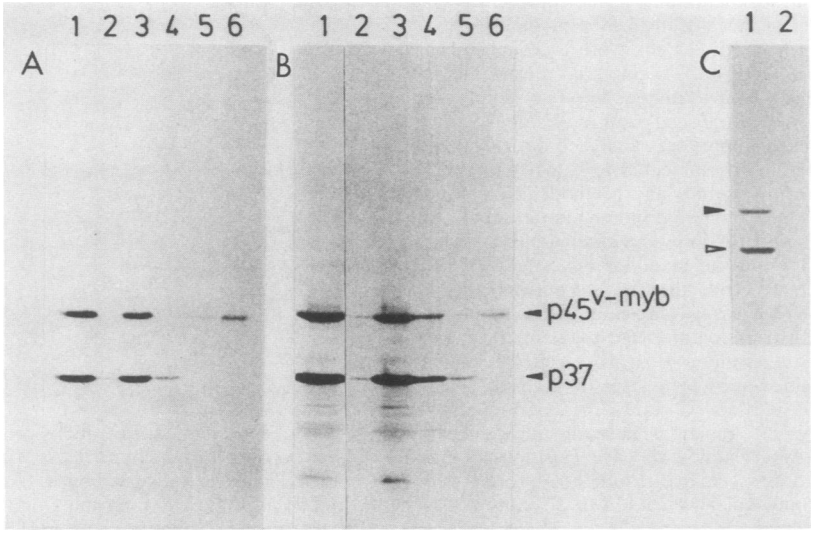

FIG. 5. Sequential extraction of $p45^{v\text{-}myb}$ from nuclei of AMV-transformed myeloblasts. (A) Nuclei (3×10^6) from BM2 cells were analyzed directly (lane 1) or were incubated sequentially in hypotonic buffer (lane 2) and hypotonic buffer supplemented with 0.4 M NaCl (lane 3) or 100 μg of DNase I per ml (lane 4) or 2 M NaCl (lane 5) to obtain a final pellet of residual nuclei (lane 6). All incubations were for 10 min at 0°C, except DNaseI treatment, which was performed at 37°C. All fractions were analyzed by immunoblotting. (B) Nuclei (5×10^6) from BM2 cells were analyzed directly (lane 1) or were extracted sequentially as in panel A, except that the order of the nuclease digestion (lane 3) and the 0.4 M NaCl extraction (lane 4) steps was reversed relative to panel A. The other lanes are as in panel A. All incubations were for 10 min at 0°C. (C) Nuclei (2×10^6) from BM2 cells were incubated in hypotonic buffer containing 0.1% SDS. The incubation was for 10 min at 0°C. High-molecular-weight DNA released from the nuclei was sheared with a syringe before separating the supernatant (lane 1) from the pellet (lane 2). The positions of $p45^{v\text{-}myb}$ and p37 are marked in panels A and B. In panel C the closed arrow indicates the position of $p45^{v\text{-}myb}$, and the open arrow marks the position of p37.

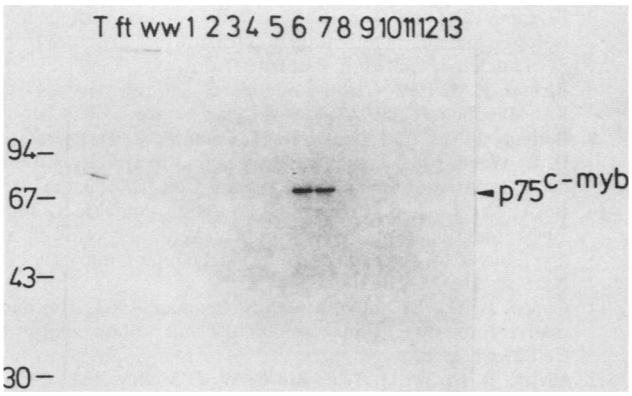

FIG. 6. DNA-cellulose chromatography of $p45^{v\text{-}myb}$. Nuclei from approximately 10^7 [^{35}S]methionine-labeled BM2 cells were incubated in the presence of 0.4 M NaCl. One-quarter of the extracted protein was analyzed directly by immunoprecipitation (lane T). The remaining protein was chromatographed on native calf thymus DNA-cellulose. ft and w mark flowthrough and wash fractions, respectively. Numbers on top refer to individual fractions of an NaCl gradient. The concentration of NaCl in fraction 6 is approximately 300 mM. The arrow marks the position of $p45^{v\text{-}myb}$, and the numbers at the side refer to the sizes (in kilodaltons) and positions of molecular size markers.

DISCUSSION

As a first step in the identification of nuclear targets involved in transformation by v-*myb* and in c-*myb* function, we determined the subnuclear location of viral and cellular *myb* proteins. Our results show that these proteins appear to interact with two distinct nuclear substructures. A large portion (80 to 90%) of the $p45^{v\text{-}myb}$ present in nuclei of AMV-transformed myeloblasts was bound to chromatin in a salt-labile manner. By contrast, a minor fraction of 10 to 20% of the nuclear $p45^{v\text{-}myb}$ presumably interacts with the nuclear matrix since it is resistant to extraction by nuclease or high salt concentrations (2 M NaCl) (5, 7, 11, 13, 18, 20, 42). We reproducibly observed the existence of two fractions of $p45^{v\text{-}myb}$ under a variety of experimental conditions, such as fractionation of nuclei in the presence of a reducing agent (dithioerythrol) or a nonionic detergent (Nonidet P-40) or in the absence of magnesium ions (unpublished data).

$p45^{v\text{-}myb}$ appears to possess DNA-binding activity; it is therefore possible that this activity is involved in the interaction of the *myb* protein with chromatin. We observed, however, that $p45^{v\text{-}myb}$ is eluted from nuclei at slightly lower salt concentrations than from DNA-cellulose. This observation might indicate that binding of $p45^{v\text{-}myb}$ to chromatin involves interactions with other chromatin proteins as well. We do not know whether binding of $p45^{v\text{-}myb}$ to the nuclear matrix is mediated by DNA, since our crude nuclear matrix preparations still contain small amounts (approximately 2 to 5%) of residual cellular DNA. However, it is quite clear that if nonextractable $p45^{v\text{-}myb}$ is bound to the residual DNA, the DNA-binding activity of the matrix fraction of the viral *myb* protein must be much less dependent on the salt concentration than the DNA-binding activity of salt-extractable $p45^{v\text{-}myb}$ and may be related to the tight DNA-binding activity of certain nuclear matrix proteins (9, 35). Alternatively, association of viral *myb* protein with the nuclear matrix might be mediated by a domain of $p45^{v\text{-}myb}$ not involved in DNA binding.

$p45^{v\text{-}myb}$ is a truncated and mutated version of the protein encoded by c-*myb* (26, 27). Our results show that the differences between $p45^{v\text{-}myb}$ and $p75^{c\text{-}myb}$ do not grossly affect the subnuclear distribution of both proteins. Thus, the domains involved in the interaction with chromatin and nuclear matrix presumably are encoded by the portion of c-*myb* that is represented in v-*myb*.

Multiple interactions of transforming proteins with nuclear components, suggesting the existence of multiple targets for these proteins, have been described previously for the simian virus 40 large T antigen (41). In that case only a minor fraction of the transforming protein interacted with chromatin. Viral *myb* protein, therefore, is the first example of a nuclear transforming protein that predominantly interacts with chromatin. Our results contrast with those of Eisenman et al. (18) showing that the proteins encoded by viral or cellular versions of the *myc* oncogene interact mainly with the nuclear matrix. However, in a recent analysis, tight interaction of *myc* proteins with the nuclear matrix was only observed when cells or isolated nuclei were incubated at elevated temperatures, suggesting that extensive interaction of *myc* proteins with the nuclear matrix might be an artifact (G. Evan, personal communication). Our own preliminary results with AMV-transformed myeloblasts that have been superinfected with MC29 virus suggest that $p45^{v\text{-}myb}$ as well as $p110^{gag\text{-}myc}$ associate primarily with the chromatin in these cells (unpublished data).

The possible functions of the subpopulations of *myb* protein that are bound to different nuclear substructures remain largely obscure. Both chromatin- and matrix-bound forms of $p45^{v\text{-}myb}$ and $p75^{c\text{-}myb}$ turn over very rapidly with half-lives of less than 1 h (unpublished data). This suggests that these proteins have regulatory functions, for example,

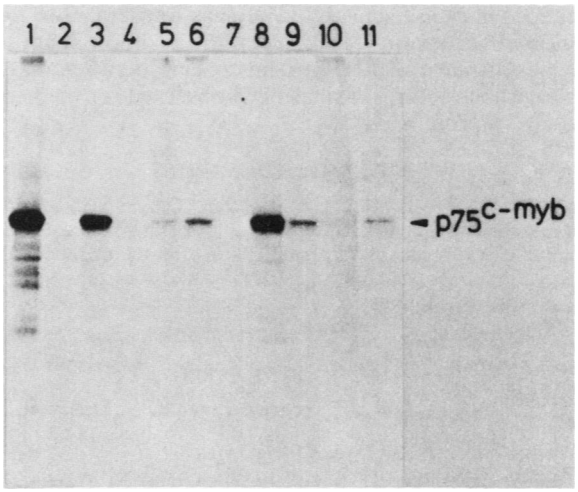

FIG. 7. Sequential extraction of $p75^{c\text{-}myb}$ from nuclei of AEV-transformed erythroblasts. Nuclei (10^7) isolated from [^{35}S]methionine-labeled AEV-transformed erythroblasts were analyzed by immunoprecipitation without fractionation (lane 1) or were incubated sequentially as described in the legend to Fig. 5 in hypotonic buffer (lane 2) and hypotonic buffer supplemented with 0.4 M NaCl (lane 3) or 100 μg of DNase I per ml (lane 4) or 2 M NaCl (lane 5) to obtain a final pellet of residual nuclei (lane 6). In parallel, 10^7 nuclei were extracted sequentially as before except that the order of the nuclease digestion (lane 8) and the 0.4 M NaCl extraction (lane 9) steps was reversed. The other lanes show extraction in hypotonic buffer (lane 7) and in hypotonic buffer supplemented with 2 M NaCl (lane 10). The final pellet of residual nuclei is analyzed in lane 11. All fractions were analyzed by immunoprecipitation. The arrow marks the position of $p75^{c\text{-}myb}$.

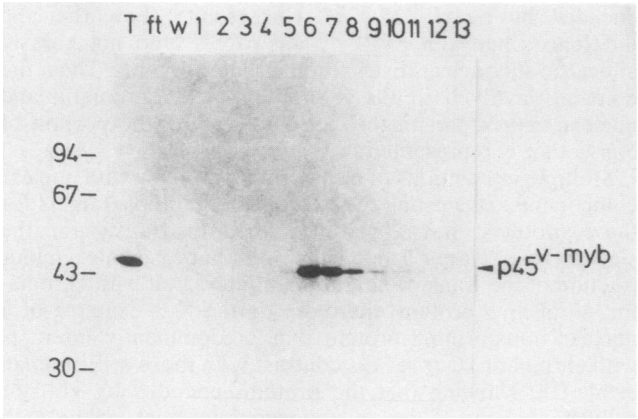

FIG. 8. DNA-cellulose chromatography of $p75^{c\text{-}myb}$. Approximately 3×10^7 nuclei prepared from [^{35}S]methionine-labeled AEV-transformed erythroblasts were incubated in the presence of 0.4 M NaCl. One-fifth of the extracted protein was analyzed directly by immunoprecipitation (lane T), and the remaining protein was chromatographed on native calf thymus DNA-cellulose. ft and w refer to the flowthrough and wash fractions, respectively. Numbers on top mark individual fractions of an NaCl gradient. The concentration of NaCl in fraction 6 is approximately 300 mM. The arrow marks the position of $p75^{c\text{-}myb}$, and the numbers at the side mark the sizes (in kilodaltons) and positions of molecular size markers.

in processes that appear to be organized around the nuclear matrix, such as the replication or the transcription of genes (2, 4, 6, 12, 14, 30, 33, 36, 44). Interestingly, chromatin-bound $p45^{v\text{-}myb}$ is attached to chromatin fragments that are slightly smaller than fragments of bulk chromatin, suggesting that $p45^{v\text{-}myb}$ is bound to chromatin regions that exhibit enhanced nuclease sensitivity, such as transcribed or newly replicated chromatin (21, 37, 38, 46). Finally, it is not clear whether a transit of *myb* proteins occurs between different nuclear substructures, as has been observed for the herpesvirus ICP8 protein (34).

ACKNOWLEDGMENTS

We thank Mike Bishop, Gerard Evan, Giovannella and Carlo Moscovici, and Thomas Graf for providing cell lines, Gerard Evan for communicating results before publication, and the members of our research group for valuable suggestions and discussions.

This work was supported by a grant from the Bundesministerium für Forschung und Technologie (BCT 0364/1).

LITERATURE CITED

1. **Abrams, H. D., L. R. Rohrschneider, and R. N. Eisenman.** 1982. Nuclear location of the putative transforming protein of avian myelocytomatosis virus. Cell **29**:427–439.
2. **Aelen, J. M A., R. J. G. Opstelten, and F. Wanka.** 1983. Organization of DNA replication in *Physarum polycephalum*. Attachment of origins of replicons and replication forks to the nuclear matrix. Nucleic Acids Res. **11**:1181–1195.
3. **Alitalo, K., G. Ramsay, J. M. Bishop, S. O. Pfeifer, W. W. Colby, and A. D. Levinson.** 1983. Identification of nuclear proteins encoded by viral and cellular *myc* oncogenes. Nature (London) **306**:274–277.
4. **Berezney, R., and L. A. Buchholtz.** 1981. Dynamic association of replicating DNA fragments with the nuclear matrix of regenerating liver. Exp. Cell Res. **123**:1–13.
5. **Berezney, R., and D. S. Coffey.** 1974. Identification of a nuclear protein matrix. Biochem. Biophys. Res. Commun. **60**: 1410–1419.
6. **Berezney, R., and D. S. Coffey.** 1975. Nuclear protein matrix: association with newly synthesized DNA. Science **189**:291–293.
7. **Berezney, R., and D. S. Coffey.** 1975. Nuclear protein matrix: isolation and characterization of a framework structure from rat liver nuclei. J. Cell Biol. **73**:616–637.
8. **Bishop, J. M.** 1983. Cellular oncogenes and retroviruses. Annu. Rev. Biochem. **52**:301–354.
9. **Bodnar, J. W., C. J. Jones, D. H. Coombs, G. D. Pearson, and D. C. Ward.** 1983. Proteins tightly bound to HeLa cell DNA at nuclear matrix attachment sites. Mol. Cell. Biol. **3**:1567–1579.
10. **Boyle, W. J., J. S. Lipsick, E. P. Reddy, and M. A. Baluda.** 1983. Identification of the leukemogenic protein of avian myeloblastosis virus and of its normal cellular homologue. Proc. Natl. Acad. Sci. USA **80**:2834–2838.
11. **Capco, D. G., K. M. Wan, and S. Penman.** 1982. The nuclear matrix: three-dimensional architecture and protein composition. Cell **29**:847–858.
12. **Ciejek, E. M., M.-J. Tsai, and B. W. O'Malley.** 1983. Actively transcribed genes are associated with the nuclear matrix. Nature (London) **306**:607–609.
13. **Comings, D. E., and T. A. Okada.** 1976. Nuclear proteins. III. The fibrillar nature of the nuclear matrix. Exp. Cell Res. **103**:341–360.
14. **Cook, P. R., J. Lang, A. Hayday, L. Lania, M. Fried, D. J. Chiswell, and J. A. Wyke.** 1982. Active viral genes in transformed cells lie close to the nuclear cage. EMBO J. **1**:447–452.
15. **Curran, T., A. D. Miller, L. Zokas, and I. Verma.** 1984. Viral and cellular *fos* proteins: a comparative analysis. Cell **36**: 259–268.
16. **Donner, P., I. Greiser-Wilke, and K. Moelling.** 1982. Nuclear localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature (London) **296**:262–266.
17. **Duesberg, P. H., K. Bister, and C. Moscovici.** 1980. Genetic structure of avian myeloblastosis virus, released from transformed myeloblasts as a defective virus particle. Proc. Natl. Acad. Sci. USA **77**:5120–5124.
18. **Eisenman, R. N., C. Y. Tachibana, H. D. Abrams, and S. R. Hann.** 1985. v-*myc*- and c-*myc*-encoded proteins are associated with the nuclear matrix. Mol. Cell. Biol. **5**:114–126.
19. **Evan, G. I., G. K. Lewis, and J. M. Bishop.** 1984. Isolation of monoclonal antibodies specific for products of avian oncogene *myb*. Mol. Cell. Biol. **4**:2843–2850.
20. **Fisher, P. A., M. Berrios, and G. Blobel.** 1982. Isolation and characterization of a proteinaceous subnuclear fraction composed of nuclear matrix, peripheral lamina and nuclear pore complexes from embryos of *Drosophila melanogaster*. J. Cell Biol. **92**:674–686.
21. **Garel, A., and R. Axel.** 1976. Selective digestion of transcriptionally active ovalbumin genes from oviduct nuclei. Proc. Natl. Acad. Sci. USA **73**:3966–3970.
22. **Gonda, T. J., and J. M. Bishop.** 1983. Structure and transcription of the cellular homolog (c-*myb*) of the avian myeloblastosis virus transforming gene (v-*myb*). J. Virol. **46**:212–220.
23. **Gonda, T. J., D. K. Sheiness, and J. M. Bishop.** 1982. Transcripts from the cellular homologs of retroviral oncogenes: distribution among chicken tissues. Mol. Cell. Biol. **2**:617–624.
24. **Gonda, T. J., D. K. Sheiness, L. Fanshier, J. M. Bishop, C. Moscovici, and M. G. Moscovici.** 1981. The genome and the intracellular RNAs of avian myeloblastosis virus. Cell **23**:279–290.
25. **Heldin, C. H., and B. Westermark.** 1984. Growth factors: mechanism of action and relation to oncogenes. Cell **37**:9–20.
26. **Klempnauer, K.-H., T. J. Gonda, and J. M. Bishop.** 1982. Nucleotide sequence of the retroviral leukemia gene v-*myb* and its progenitor c-*myb*: the architecture of a transduced oncogene. Cell **31**:453–463.
27. **Klempnauer, K.-H., G. Ramsay, J. M. Bishop, M. G. Moscovici, C. Moscovici, J. P. McGrath, and A. D. Levinson.** 1983. The product of the retroviral transforming gene v-*myb* is a truncated version of the protein encoded by the cellular oncogene c-*myb*. Cell **33**:345–355.
28. **Klempnauer, K.-H., G. Symonds, G. I. Evan, and J. M. Bishop.** 1984. Subcellular localization of proteins encoded by oncogenes of avian myeloblastosis virus and avian leukemia virus E26 and by the chicken c-*myb* gene. Cell **37**:537–547.

29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Mariman, E. C. M., C. A. G. van Eekelen, R. J. Reinders, A. J. M. Berns, and W. J. van Venrooij.** 1982. Adenovirus heterogeneous nuclear RNA is associated with the host nuclear matrix during splicing. J. Mol. Biol. **154**:103–119.
31. **Moscovici, C.** 1975. Leukemic transformation with avian myeloblastosis virus: present status. Curr. Top. Microbiol. Immunol. **71**:79–101.
32. **Moscovici, C., N. Zeller, and M. G. Moscovici.** 1982. Continuous lines of AMV-transformed non-producer cells: growth and oncogenic potential in the chick embryo, p. 435–449. *In* R. F. Revoltella, G. M. Pontieri, C. Basilico, G. Rovera, R. C. Gallo, and J. H. Subak-Sharpe (ed.), Expression of differentiated functions in cancer cells. Raven Press, New York.
33. **Pardoll, D. M., B. Vogelstein, and D. S. Coffey.** 1980. A fixed site of DNA replication in eucaryotic cells. Cell **19**:527–536.
34. **Quinlan, M. P., L. B. Chen, and D. M. Knipe.** 1984. The intranuclear distribution of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell **36**:857–868.
35. **Razin, S. V., V. V. Chernokhvostov, A. V. Roodyn, I. B. Zbarsky, and G. P. Georgiev.** 1981. Proteins tightly bound to DNA in the regions of DNA attachment to the skeletal structures of interphase nuclei and metaphase chromosomes. Cell **27**:65–73.
36. **Robinson, S. I., B. D. Nelkin, and B. Vogelstein.** 1982. The ovalbumin gene is associated with the nuclear matrix of chicken oviduct cells. Cell **28**:99–106.
37. **Seale, R. L.** 1975. Assembly of DNA and protein during replication in HeLa cells. Nature (London) **225**:247–249.
38. **Seale, R. L.** 1978. Nucleosomes associated with newly replicated DNA have an altered conformation. Proc. Natl. Acad. Sci. USA **75**:2717–2721.
39. **Sheiness, D., and M. Gardinier.** 1984. Expression of a proto-oncogene (proto *myb*) in hematopoietic tissues of mice. Mol. Cell. Biol. **4**:1206–1212.
40. **Souza, L. M., J. N. Strommer, R. L. Hilyard, M. C. Komaromy, and M. A. Baluda.** 1980. Cellular sequences are present in the presumptive avian myeloblastosis virus genome. Proc. Natl. Acad. Sci. USA **77**:5177–5181.
41. **Staufenbiel, M., and W. Deppert.** 1983. Different structural systems of the nucleus are targets for SV40 large T antigen. Cell **33**:173–181.
42. **Staufenbiel, M., and W. Deppert.** 1984. Preparation of nuclear matrices from cultured cells: subfractionation of nuclei in situ. J. Cell Biol. **98**:1886–1894.
43. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
44. **van Eekelen, C. A. G., and W. J. van Venrooij.** 1981. hnRNA and its attachment to a nuclear protein matrix. J. Cell Biol. **88**:554–563.
45. **Weintraub, H.** 1984. Histone-H1-dependent chromatin superstructures and the suppression of gene activity. Cell **38**:17–27.
46. **Weintraub, H., and M. Groudine.** 1976. Chromosomal subunits in active genes have an altered conformation. Science **93**: 848–858.