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Biochemistry

Decreased DNA-binding ability of purified transformation-specific proteins from deletion mutants of the acute avian leukemia virus MC29

(transformation mechanisms/monoclonal antibodies/bone marrow cells and fibroblasts/avian erythroblastosis virus/Fujinami sarcoma virus)

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ABSTRACT **Avian myelocytomatosis virus MC29 is a highly oncogenic replication-defective retrovirus that predominantly affects hematopoietic cells and causes acute leukemia *in vivo* and that transforms hematopoietic cells as well as fibroblasts *in vitro*. The transformation-specific sequence, v-*myc*, is expressed as part of a fusion protein that contains the viral structural protein p19. By use of monoclonal antibodies against p19 we showed that the v-*myc*-encoded protein is located in the nucleus of MC29-transformed fibroblasts and that after purification over an immunoaffinity column the protein binds to double-stranded DNA. In this report we describe the analysis of the v-*myc* gene product from MC29-transformed bone marrow cells. The immunoaffinity column-purified protein from these cells also bound to DNA and was indistinguishable from the purified protein from MC29-transformed fibroblasts. In addition, the v-*myc* gene products from fibroblasts transformed by three nonconditional mutants of MC29—which transform hematopoietic cells with a markedly decreased efficiency *in vivo* and *in vitro* but still transform fibroblasts *in vitro*, expressing deleted v-*myc* proteins—were analyzed. In contrast to the wild-type protein, the purified mutant proteins had decreased DNA-binding abilities. Furthermore, a preferential binding of the wild-type protein to poly(dG)·poly(dC) duplexes was observed. Such a specificity was lost with a mutant protein. These results provide evidence that the interaction of the v-*myc* protein with DNA may be directly involved in transformation of the hematopoietic target cells. Further, the transformation-specific fusion proteins purified from cells transformed by avian erythroblastosis virus, which belongs to a different class of acute leukemia viruses, and by Fujinami sarcoma virus were found not to be DNA-binding proteins, suggesting the existence of different transformation mechanisms.**

In cells transformed by avian myelocytomatosis virus MC29, the v-*myc* gene is expressed as a fusion protein that contains a transformation-specific region and the structural protein p19, which is the NH_2-terminal region of the gag protein (1). The protein has a M_r of 110,000 and is designated as $p110^{gag-myc}$ (2). We have previously studied the $p110^{gag-myc}$ protein by using monoclonal antibodies against p19 (3, 4). The protein is located in the nucleus of MC29-transformed nonproducer quail fibroblasts and, after purification, binds to double-stranded (ds) DNA (4). Because MC29 virus predominantly affects bone marrow cells *in vivo*, these are considered to be the actual target cells (5). Therefore, it was of interest to analyze the $p110^{gag-myc}$ protein from bone marrow cells and compare it to the protein from a fibroblast cell line, both transformed by MC29. This is of particular importance because three deletion mutants of MC29 have been isolated which reveal a potential of transformation of hematopoietic cells *in vivo* and *in vitro* that was decreased to 1/100th. However, they still transform fibroblasts *in vitro* with the same efficiency as the wild-type virus (6). The deletion mutants were isolated from an MC29-transformed quail fibroblast cell line, Q10, where they had generated spontaneously (7, 8). The three nonproducer quail fibroblast clones Q10A, Q10C, and Q10H continuously synthesize gag-fusion proteins of M_rs 100,000, 95,000, and 90,000 ($p100^{gag-myc}$, $p95^{gag-myc}$, and $p90^{gag-myc}$), respectively (6, 7). All three proteins were shown to have deletions in the *myc*-specific region (7, 8).

Further, two other transformation-specific proteins were analyzed, which were from cells transformed by viruses belonging to different classes than MC29 but were also gag-fusion proteins. One of them, the avian erythroblastosis virus (AEV), is an acute leukemia virus that induces acute erythroleukemia and sarcomas *in vivo* and transforms bone marrow cells and fibroblasts *in vitro* (5). In contrast to MC29, cells transformed by AEV express two presumptive transformation-specific proteins. The first one, $p75^{gag-erbA}$, is fused to gag proteins, including p19, whereas the second protein, $p61^{erbB}$, is expressed without gag proteins (9). The role of these two proteins is still unclear. Fluorescence microscopy of AEV-transformed nonproducer chicken fibroblasts with monoclonal or rabbit antibodies against p19 gave rise to cytoplasmic fluorescence with no indication of a distinct nuclear fluorescence in contrast to MC29-transformed quail fibroblasts (10, 11). The third gag-fusion protein analyzed here was that of Fujinami sarcoma virus (FSV)-transformed rat cells (12). This virus is replication-defective and causes solid tumors *in vivo* and transforms fibroblasts *in vitro* (13). The transformation-specific protein, $p130^{gag-fps}$, was found to be closely associated with a protein kinase (12), thus resembling the protein from Rous sarcoma virus-transformed cells, $pp60^{src}$ (14).

The transformation-specific proteins from MC29 wild-type and mutant cells and from AEV- and FSV-transformed cells were purified by immunoaffinity column chromatography and the DNA-binding abilities of the purified proteins were analyzed. Although MC29 wild-type $p110^{gag-myc}$ was a DNA-binding protein (4), the $p100^{gag-myc}$, $p95^{gag-myc}$, and $p90^{gag-myc}$ from the MC29 deletion mutants had decreased DNA-binding abilities, and the $p75^{gag-erbA}$ from AEV- and the $p130^{gag-fps}$ proteins from FSV-transformed cells did not bind to DNA.

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Abbreviations: AEV, avian erythroblastosis virus; FSV, Fujinami sarcoma virus; SV40, simian virus 40; ds, double-stranded.

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MATERIALS AND METHODS

Cells. MC29-Q8-NP is an established nonproducer MC29-transformed quail fibroblast cell line (1). The nonproducer cell lines Q10A, Q10C, and Q10H are quail fibroblasts transformed by three deletion mutants of MC29 (6). They were obtained from M. Hayman (London). MC29-transformed bone marrow cells were a gift from H. Beug (Heidelberg). A nonproducer clone, MC29-BM cl 10-NP, was selected in this laboratory. AEV cl 23 is a nonproducer AEV-transformed established chicken fibroblast cell line supplied by S. Martin (Berkeley, CA). All cells were grown in Dulbecco's modified Eagle's medium with 10% tryptose phosphate broth/10 mM Hepes/5% fetal calf serum/2% heat-inactivated chicken serum/0.5% dimethylsulfoxide. FSV cl 9 is a FSV-transformed rat fibroblast cell line obtained from H. Hanafusa (New York). It was grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum.

Radioactive Labeling of Cells. Cells were labeled for 2 hr with methionine-free medium supplemented with 250 μCi (1 Ci = 3.7×10^{10} Bq) of [^{35}S]methionine per ml. After washing the cells with phosphate-buffered saline (17 mM Na_2PO_4/2.6 mM KH_2PO_4, pH 7.4/120 mM NaCl) they were lysed with 2 ml of Ripa buffer (50 mM Tris·HCl, pH 7.2/150 mM NaCl/0.1% $NaDodSO_4$/1% sodium deoxycholate/1% Triton X-100) per dish. Bone marrow cells that grew in suspension were radioactively labeled in Eppendorf tubes (10^8 cells in 0.5 ml of [^{35}S]methionine-containing medium).

Immunoaffinity Column Chromatography. Details of the procedure have been described (4). Briefly, monoclonal anti-p19 IgG was covalently coupled to protein A-Sepharose CL-4B (Pharmacia) according to published procedures (15). The material was used for a 0.6 × 1 cm column. Cells from six Petri dishes lysed in Ripa buffer were applied to the column. After extensive washing—first with Ripa buffer, then with 50 mM Tris·HCl, pH 7.4/1 M NaCl/1% Triton X-100, and finally with phosphate-buffered saline—the protein was eluted with a buffer at pH 2 containing 100 mM citric acid, 300 mM NaCl, 50% ethyleneglycol, and 0.1% Triton X-100. Fractions of 1 ml were collected. The purity of the protein was tested on a 10% $NaDodSO_4$/polyacrylamide gel (16).

DNA Filter-Binding Assay. Details of the procedure have been described (4). The purified proteins were standardized to equal amounts by determining the acid-precipitable radioactivity and the protein content of the original lysate. Increasing amounts of purified protein (30,000 cpm/μg) in up to 50 μl were incubated with 2 μg of ds [^{3}H]DNA from chicken embryo fibroblasts (8,000 cpm/μg), which was sheared to about 10 kilobases. When salt dependence was analyzed, the incubation mixture and the washing buffer contained the salt concentrations indicated. Subsequently, the filters were washed in addition with the 50 mM NaCl-containing washing buffer. The amount of radioactive DNA bound to the filter was determined.

Binding of Native Proteins to DNA. Five milliliters of MC29-Q8-NP lysate (1.7 mg/ml) and 3.5 ml of Q10C lysate (2.6 mg/ml) were used to bind the wild-type and mutant proteins to immunoaffinity columns. The columns were washed but not treated with elution buffer. Instead, 3×10^5 cpm of nick-translated simian virus 40 (SV40) DNA (1.7×10^6 cpm/μg) was applied to each column and incubated for 30 min at room temperature. After several washes with TE buffer (50 mM Tris·HCl, pH 8/2 mM EDTA) to remove nonspecifically bound DNA, the column material was divided into four aliquots, which were washed with 1 ml of TE buffer containing 0, 50, 100, and 200 mM NaCl, respectively. This treatment was repeated three times. The residual DNA–protein complexes were then collected by low-speed centrifugation and processed for gel electrophoresis on 1.5% agarose gels. The gels were exposed for autoradiography and analyzed by laser densitometry (LKB, Ultroscan 2202).

DNA–Protein Cosedimentation Analysis. Protein and DNA (50 μg) were mixed under the same conditions as described for the filter-binding assay in a total volume of 500 μl and were sedimented through a 10–30% glycerol gradient in a Beckman Ti SW 41 rotor at 30,000 rpm for 19 hr at 4°C. Five-hundred-microliter fractions were collected. The DNA content of each fraction was determined by measuring the absorbance at 260 nm (4).

Isolation of [^{3}H]DNA. Chicken embryo fibroblasts were labeled for 12 hr with 10 μCi of [^{3}H]thymidine per ml of growth medium. DNA was isolated by standard procedures (17).

Binding to Synthetic Polymers. Purified wild-type and mutant proteins were incubated with chicken fibroblast ds [^{3}H]DNA under conditions of a filter-binding assay. The amounts of protein and DNA were adjusted so that 50% of the DNA bound by the wild-type protein, corresponding to 1 μg, was 30,000 cpm per assay and was designated as 100%. The amount of DNA bound by the mutant protein was 5,000 cpm per assay, corresponding to 100%. For competition the incubation mixtures were supplemented with increasing amounts of synthetic poly(dA)·poly(dT) or poly(dG)·poly(dC) duplexes (Boehringer Mannheim). The amount of [^{3}H]DNA bound to the filter was determined.

RESULTS

Comparison of the v-myc Proteins from Bone Marrow Cells and Fibroblasts. Recently we described the DNA-binding properties of the $p110^{gag-myc}$ protein purified from MC29-Q8-NP cells, an established nonproducer cell line of quail fibroblasts transformed by MC29 (1). Because MC29 primarily affects hematopoietic cells and not fibroblasts *in vivo*, we wanted to exclude the possibility that the v-*myc* gene products expressed by the transformed fibroblasts and by bone marrow cells were different. Therefore, chicken bone marrow cells were infected *in vitro* with various dilutions of a virus stock of MC29 with RAV-1 as helper virus. After soft-agar cloning, the nonproducer clone MC29-BM cl 10 was selected and used for further studies. The cells were labeled with [^{35}S]methionine and the $p110^{gag-myc}$ was purified by immunoaffinity column chromatography by using monoclonal antibodies against p19 as described for the protein from MC29-Q8-NP fibroblasts (3, 4). The result of the purification of the proteins both from bone marrow cells and fibroblasts is shown in Fig. 1 *Upper*. The purification achieved was about 3,000-fold, and the proteins from both sources had the same molecular weights. The ability of both proteins to bind to DNA was compared in the filter binding assay. Both proteins bound to ds DNA with similar efficiencies. The result is shown in Fig. 2.

v-myc Proteins from Cells Transformed by MC29 Deletion Mutants. All analyses of DNA–protein interaction with the purified v-*myc* gene products *in vitro* thus far lacked the direct evidence that DNA binding was involved in the transformation process. To look for a correlation between DNA binding and transformation, the deleted v-*myc* gene products from quail fibroblast cell lines transformed by the three deletion mutants from MC29 were purified by immunoaffinity column chromatography. The results are shown in Fig. 1 *Lower*. The purified proteins from cells transformed with the three mutants and with the wild-type viruses were analyzed simultaneously in a filter-binding assay for their ability to bind to DNA. Compared to the wild-type protein, the proteins from all three deletion mutants had drastically decreased DNA-binding capacities (Fig. 3A). The amount of DNA bound by mutant proteins was about 1/20th as much if compared to the wild type. However,

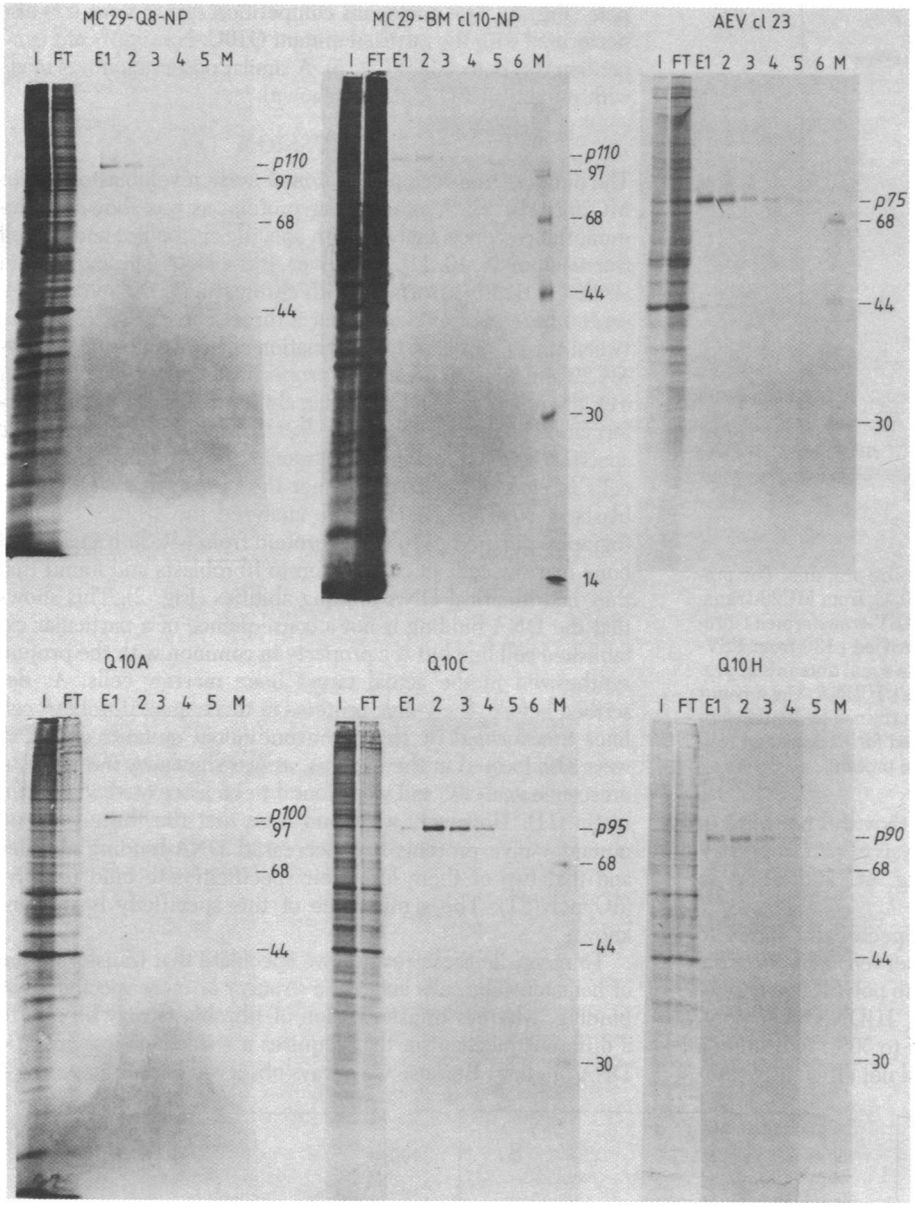

FIG. 1. Purification of transformation-specific proteins. The transformation-specific proteins from MC29-Q8-NP quail fibroblasts, from MC29-BM cl 10-NP bone marrow cells, from AEV cl 23 chicken fibroblasts, and from quail fibroblast cell lines Q10A, Q10C, and Q10H, transformed by mutants of MC29, were purified from [^{35}S]methionine-labeled cells by immunoaffinity column chromatography by using monoclonal IgG against p19. Five microliters of the input (I), 5 μl of the flow-through (FT), and 75 μl of the individual column fractions (E) were directly applied to 10% NaDodSO$_4$/polyacrylamide gels and exposed for autoradiography. M represents ^{14}C-labeled marker proteins.

this number depended on the concentration of NaCl during the assay (data not shown).

The DNA-binding properties of the wild-type and the mutant proteins were quantitatively compared by determining the salt concentrations for the half-maximal binding capacities in the filter-binding assay. The amounts of DNA bound by wild-type p110$^{gag-myc}$ and mutant Q10C protein p95$^{gag-myc}$ in the absence of salt were taken as 100%. Under these conditions, the amount of DNA bound by the mutant protein was 1/20th of that of the wild-type protein. The amount of DNA bound in the presence of increasing salt concentrations by both proteins is shown in Fig. 3*B*. The half-maximal binding of the wild-type protein was achieved at 180 mM, and, for the mutant protein, at 80 mM.

Because the purification procedure of the wild-type and mutant proteins involves treatment with low pH buffer, a comparison of binding of native proteins to DNA was also performed. For this purpose wild-type and mutant Q10C proteins immobilized on immunoaffinity columns were exposed to radioactively labeled SV40 DNA fragments. The amount of DNA bound to the proteins after washing procedures at 0, 50, 100 and 200 mM salt was determined. The result is shown in Fig. 3*C*. About 50% of the DNA was eluted with 50 mM salt in the case of the mutant, whereas 150 mM salt was required for the wild type. This result is in agreement with the salt concentrations determined for half-maximal binding with the purified proteins presented in Fig. 3*B*, indicating that the native and purified proteins closely resemble each other.

To support these results, a sedimentation analysis of ds DNA with purified p110$^{gag-myc}$ and p95$^{gag-myc}$ proteins was performed as described (4). For this purpose, ds DNA was mixed with each of the two proteins and sedimented through a glycerol gradient. Fig. 4 shows that the wild-type p110$^{gag-myc}$ cosedimented with the DNA, whereas the mutant p95$^{gag-myc}$ remained on top of the gradient. Proteins run without DNA stayed on top of the gradient (not shown). The wild-type protein always showed a tendency to aggregate. These aggregates migrated further to the bottom of the gradient without binding to DNA.

Transformation-Specific Proteins from Cells Transformed by Other Viruses. Immunoaffinity column chromatography was also used to purify the p75$^{gag-erbA}$ protein from a nonproducer cell line of AEV-transformed chicken fibroblasts, AEV cl 23 (Fig. 1 *Upper*), and the p130$^{gag-fps}$ from a FSV-tranformed rat fibroblast cell line, FSV cl 9. The filter-binding assay with these proteins was performed under the same conditions as described for

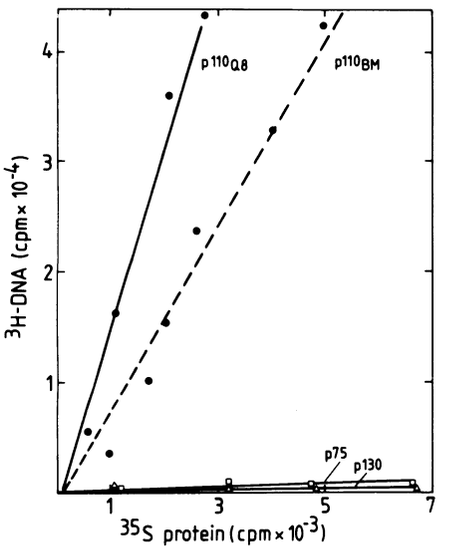

FIG. 2. DNA binding of purified transforming proteins. The proteins from MC29-transformed fibroblasts ($p110_{Q8}$), from MC29-transformed bone marrow cells ($p110_{BM}$), and from AEV-transformed fibroblasts (p75) shown in Fig. 1 *Upper* and the purified p130 from FSV-transformed rat fibroblasts were standardized to equal amounts of protein and assayed in a filter-binding assay with ds [^{3}H]DNA. The amount of radioactive DNA bound to the filter was determined by liquid scintillation spectroscopy. All values were corrected for background contributed by the DNA alone (1,000 cpm) and the protein.

the gag–myc proteins. Fig. 2 shows that they did not bind to DNA. Furthermore, a sedimentation analysis of p75 from AEV with DNA is shown for comparison in Fig. 4*C*, indicating no detectable binding as well.

Wild-Type but not Mutant Protein Specifically Binds to Poly(dG)·Poly(dC). Furthermore, we observed a preferential binding of the purified wild-type protein to poly(dG)·poly(dC). A 500-fold weight excess of chicken ds [^{3}H]DNA over poly(dG)·poly(dC) was competitively inhibited to 50%, indicating a preferential binding. Poly(dA)·poly(dT) did not efficiently compete (Fig. 5). The analogous competition experiment was also performed with the purified mutant Q10C. No significant competition was detectable (Fig. 5). A similar observation was made with mutant Q10H (data not shown).

DISCUSSION

The putative transforming protein of avian myeloblastosis virus MC29, $p110^{gag-myc}$, is a nuclear protein, as was shown by immunofluorescence analysis with specific antibodies and by cell fractionation (4, 10, 11). Moreover, it is a DNA-binding protein (4) that is tightly associated with chromatin (11). The data presented here give evidence that a direct correlation exists between the potential of transformation of hematopoietic cells by MC29 and the DNA-binding properties of the v-*myc* protein. Yet, the role of the DNA-binding ability of this protein in transformation is complicated by the fact that mutations in the v-*myc* specific regions affect only the transformation of hematopoietic cells *in vivo* and *in vitro* but not the transformation of fibroblasts *in vitro* (6, 7). Here we analyzed the affinity chromatography-purified $p110^{gag-myc}$ protein from MC29-transformed bone marrow cells in comparison to fibroblasts and found that they had identical DNA-binding abilities (Fig. 2). This shows that the DNA binding is not a consequence of a particular established cell line but is a property in common with the protein synthesized in the actual target bone marrow cells. As described before, the v-*myc* proteins in three quail fibroblast cell lines transformed by three nonconditional mutants of MC29 were also located in the nucleus, as was shown by immunofluorescence analysis, and were found to be associated with chromatin (11). However, we found here that the three purified mutant v-myc proteins had decreased DNA-binding abilities and that two of them lost their specificities to bind to poly(dG)·poly(dT). The significance of this specificity is still unknown.

To reconcile these results, we speculate that transformation of hematopoietic cells involves a stronger or more specific DNA binding, whereas transformation of fibroblasts may be due to a different mechanism that requires a weaker or less specific DNA binding. Because we always observed higher amounts of

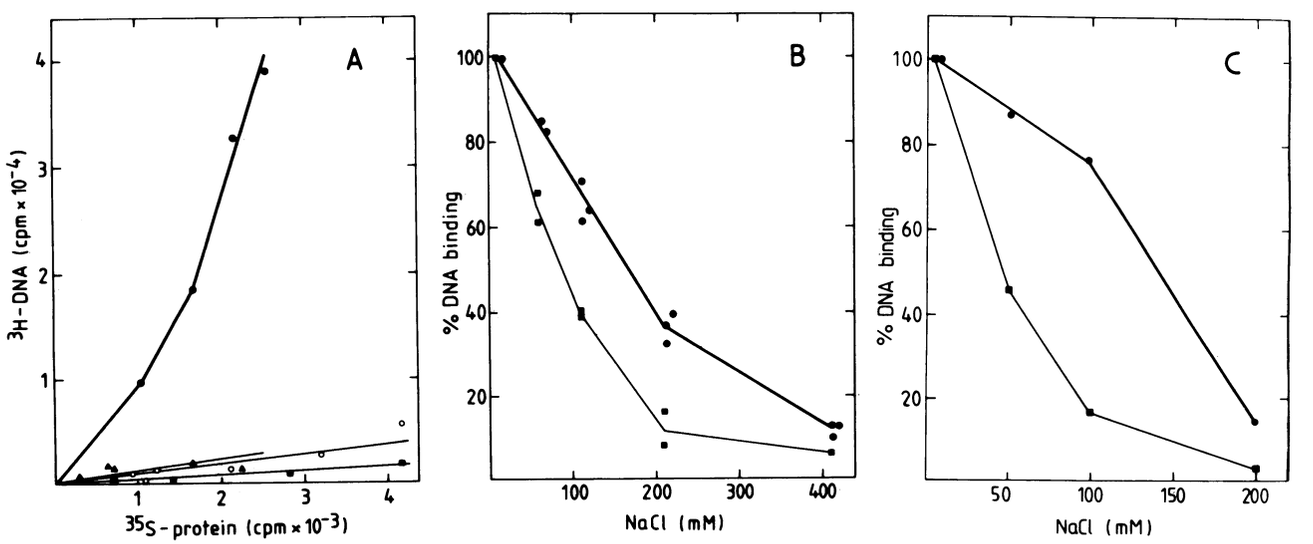

FIG. 3. (*A*) DNA binding of purified v-*myc* proteins from wild-type and mutant MC29-transformed fibroblasts. The purified proteins (Fig. 1) were tested for DNA binding in a filter-binding assay by using standardized amounts of protein. ●, $p110^{gag-myc}$ from MC29-Q8-NP; ▲, $p100^{gag-myc}$ from Q10A; ■, $p95^{gag-myc}$ from Q10C; and ○, $p90^{gag-myc}$ from Q10H. (*B*) Determination of the half-maximal binding of wild-type $p110^{gag-myc}$ (●) and mutant $p95^{gag-myc}$ (■). Filter-binding assays were performed with standardized amounts of proteins in the presence of increasing amounts of NaCl in the incubation mixture. The amount of DNA bound in the absence of salt was defined as 100% in both cases. (*C*) Binding of DNA to native proteins. Wild-type (●) and mutant Q10C (■) proteins were bound to immunoaffinity columns. The immobilized proteins were mixed with ^{32}P-labeled SV40 DNA, washed, distributed to aliquots, and treated with 0, 50, 100, and 200 mM NaCl, respectively. DNA still bound after this treatment was analyzed on agarose gels that were exposed for autoradiography and subsequently quantitated by densitometry. The SV40 DNA bound at each salt concentration was integrated and expressed as percentage relative to the amount of DNA bound in the absence of salt.

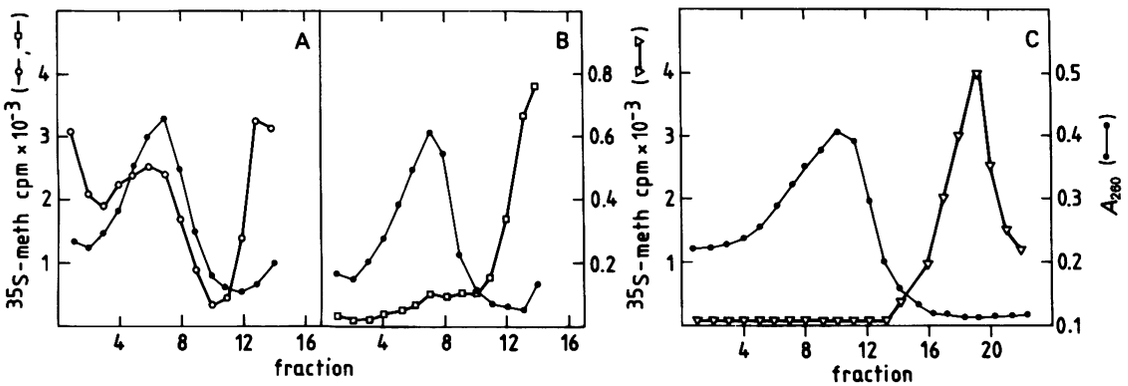

FIG. 4. Sedimentation analysis of MC29 wild-type–, mutant–, and AEV protein–DNA mixtures. The purified wild-type protein (*A*; M_r 110,000), the Q10C mutant protein (*B*; M_r 95,000), and the AEV protein (*C*; M_r 75,000) were mixed with 80 μg of cellular ds DNA each and sedimented through a 10–30% glycerol gradient. Fractions were collected from bottom to top. The amount of [^{35}S]methionine-labeled protein (^{35}S-meth) of each fraction was determined by liquid scintillation spectroscopy of 100 μl, and the amount of DNA, by absorption at 260 nm (●——●).

mutant than wild-type proteins in transformed fibroblasts (unpublished data), weaker DNA-binding ability may be compensated for by larger amounts (about 5-fold) of mutant protein and thus result in fibroblastic transformation. Other domains of the protein may also play a role. Furthermore, it is conceivable that transformation of fibroblasts involves cellular, in addition to viral, factors or mechanisms—e.g., fibroblast-specific proteins that overcome the defects of the v-*myc* deleted regions.

It has been excluded before that the binding ability of p110$^{gag-myc}$ is due to its gag portion or to other gag-related polyproteins synthesized by virus-producing cells, as these do not bind to DNA (4). Further, antibodies against p19 did not interfere with DNA binding (unpublished data). Additional evidence for the specificity of the p110$^{gag-myc}$–DNA interaction is given by the complete lack of DNA binding by p75$^{gag-erbA}$, one of the two putative AEV-transforming proteins (9), and by p130$^{gag-fps}$, the transformation-specific protein from FSV (12). Both proteins were shown to be absent from the nucleus of transformed cells (10, 11, 18). Although nothing is known about the possible transformation mechanism by AEV, due to the insufficient knowledge about the second putative transforming protein p61^{erbB} (9), a kinase has been found to be associated with p130$^{gag-fps}$ from FSV-transformed cells (13), even after purification (ref. 18; unpublished data). This suggests a transformation mechanism similar to the one from Rous sarcoma virus-

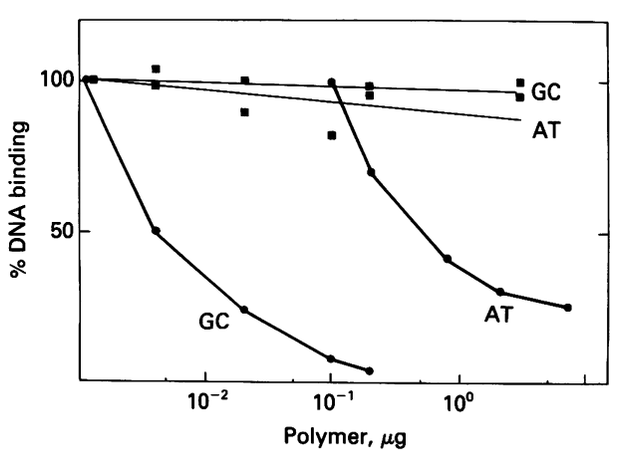

FIG. 5. Binding of poly(dG)·poly(dC) and poly(dA)·poly(dT) to wild-type and mutant proteins. Purified wild-type (●) and mutant Q10C (■) proteins were incubated with ds [^{3}H]DNA and competitively inhibited with the indicated amounts of poly(dG)·poly(dC) and poly(dA)·poly(dT) (indicated as GC and AT, respectively); 100% corresponds to 1 μg of DNA and 30,000 cpm for the wild-type and 0.4 μg of DNA and 5,000 cpm for the mutant.

transformed cells (14). In contrast, no associated kinase has been found for the purified or immune complex-bound p110$^{gag-myc}$ from MC29-transformed cells (18, 19).

From these results we conclude that at least three different mechanisms may lead to viral cell transformation. The first mechanism involves a cytoplasmic or membrane-bound protein that is associated with a kinase, as described for Rous sarcoma virus (14) and for FSV (12). The second mechanism involves a nuclear protein that binds to DNA, a property which is of possible importance for the transformation of target cells by MC29, whereas transformation of fibroblasts seems to involve other factors. A third mechanism may be responsible for transformation by AEV, which involves two putative transforming proteins, p75$^{gag-erbA}$ and p61^{erbB}, both with unknown functions.

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