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The transforming protein of the MC29-related virus CMII is a nuclear DNA-binding protein whereas MH2 codes for a cytoplasmic RNA-DNA binding polyprotein

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The acute avian leukemia viruses MH2 and CMII belong to the group of avian myelocytomatosis viruses, the prototype virus of which is MC29. This group of viruses is characterized by *myc*-specific oncogenes which are presumably expressed as gag-myc polyproteins. These polyproteins are synthesized in non-producer cells transformed by MH2 and CMII and have mol. wts. of 100 000 (p100) and 90 000 (p90), respectively. Monoclonal antibodies against the N terminus of gag, p19, were used to localize the protein in MH2- and CMII-transformed non-producer fibroblasts. Immunofluorescence and cell fractionation indicated that >90% of p100 from MH2 was located in the cytoplasm, whereas >70% of p90 from CMII resided in the nucleus. Isolation of p100 and p90 by immunoaffinity chromatography resulted in an ~2000-fold purification of the two polyproteins. Both of them, as well as p110 of MC29, bound to double-stranded DNA of chick fibroblasts *in vitro*. However, only the MH2-specific polyprotein p100 bound to RNA *in vitro*. Such a binding was not observed for p90 or p110, or for the purified gag precursor Pr76. Another polyprotein, gag-erbA, from avian erythroblastosis virus, which is also located in the cytoplasm, did not bind to RNA. Our results indicate that the CMII-specific polyprotein p90 behaved indistinguishably from the p110 of MC29. However, the MH2-specific polyprotein p100 exhibited unique and novel properties which were distinct from a gag-myc-type protein.

Key words: purified transforming proteins/monoclonal antibodies/MC29 family/exceptions from *gag-myc*

Introduction

The acute avian leukemia viruses can be classified into three groups by their oncogenic spectra as well as by homologies of their oncogenes (for reviews, see Hayman, 1981; Graf and Beug, 1978; Graf and Stehelin, 1982; Bister and Duesberg, 1982). One of these groups comprises the avian myelocytomatosis viruses MC29, the oncogene of which has been designated *myc*. Myc-related sequences have been identified in three MC29-related viruses, the Mill Hill No. 2 (MH2), the myelocytomatosis virus CMII, and Oker Blom's isolate OK10. Another group is represented by the avian erythroblastosis virus (AEV) which has two oncogenes, *erbA*, which is linked to the structural protein gag, and *erb*B (Vennström and Bishop, 1982), both of which are unrelated to *myc* (Roussell *et al.*, 1979; for review, see Bister and Duesberg, 1982). The avian myeloblastosis virus, AMV, belongs to the third group and will not be discussed here (for review, see Graf and Stehelin, 1982).

The viruses of the MC29 family induce myelocytomatosis and liver and kidney carcinomas *in vivo* (Graf and Beug, 1978) and transform fibroblasts and macrophages *in vitro* (Beug *et al.*, 1979). The sequences responsible for the oncogenicity of these viruses are presumably encoded by polyproteins, gag-myc, consisting of a portion of the gag protein covalently linked to myc, the transformation-specific protein. While the polyproteins of MC29 and CMII are of the gag-myc type, with mol. wts. of 110 000 (p110) and 90 000 (p90), respectively (for review, see Hayman, 1981), the p100 of MH2 was considered to be gag-myc mainly by analogy to the other two (Hu *et al.*, 1978; Hu and Vogt, 1979). OK10 codes for a gag-myc protein as well as a myc protein unlinked to a viral structural protein (Saule *et al.*, 1982).

While the oncogenic sequences *myc*, which are probably derived from a host cell sequence *c-myc*, were highly related in the case of MC29, CMII and OK10, less homology has been reported between MC29 and MH2 (Roussell *et al.*, 1979; Sheiness *et al.*, 1980; Duesberg and Vogt, 1979). Recently MH2 was found to be much more oncogenic than MC29 *in vivo* and it exhibits a higher efficiency of transformation in quail fibroblasts *in vitro* (Linial, 1982) suggesting some more distant relationship between the two. Since we have previously analyzed the p110 gag-myc polyprotein from MC29, we were interested in comparing its properties with the polyproteins of the other viruses belonging to the MC29 family.

The p110 of MC29 was recently characterized by monoclonal antibodies against p19, the N terminus of the gag protein. p110 was found to be located in the nucleus (Donner *et al.*, 1982; Adams *et al.*, 1982). It is closely associated with chromatin in non-producer transformed fibroblasts as well as bone marrow cells, as judged by cell fractionation and immunofluorescence (Bunte *et al.*, 1982). The protein, if purified by immunoaffinity column, binds to double-stranded DNA *in vitro* (Donner *et al.*, 1982). Deletion mutants of MC29, with altered transforming properties *in vivo* (Ramsay *et al.*, 1980), exhibit reduced DNA-binding abilities *in vitro* (Donner *et al.*, 1983).

Here we present an analysis of the gag-linked polyproteins of the two MC29-related viruses MH2 and CMII. We have studied their intracellular localizations in non-producer fibroblasts and their properties after purification. While the polyprotein p90 of CMII behaved very similarly to the gag-myc protein of MC29, the p100 of MH2 exhibited distinct properties. Our data indicate that p100 is not a typical gag-myc protein.

Results

Two established non-producer cell lines, MH2-transformed quail fibroblasts, MH2-NP, and CMII-transformed quail fibroblasts, CMII-NP, which express virus-specific transforming proteins but do not produce virus, were analyzed throughout this study to guarantee the absence of gag-related proteins other than the gag-myc transforming proteins. The localization of the gag-related polyproteins, p100 from MH2-transformed cells and p90 from CMII-transformed cells, were analyzed by indirect immunofluorescence using monoclonal antibodies against p19, the N-terminal portion of the gag pro-

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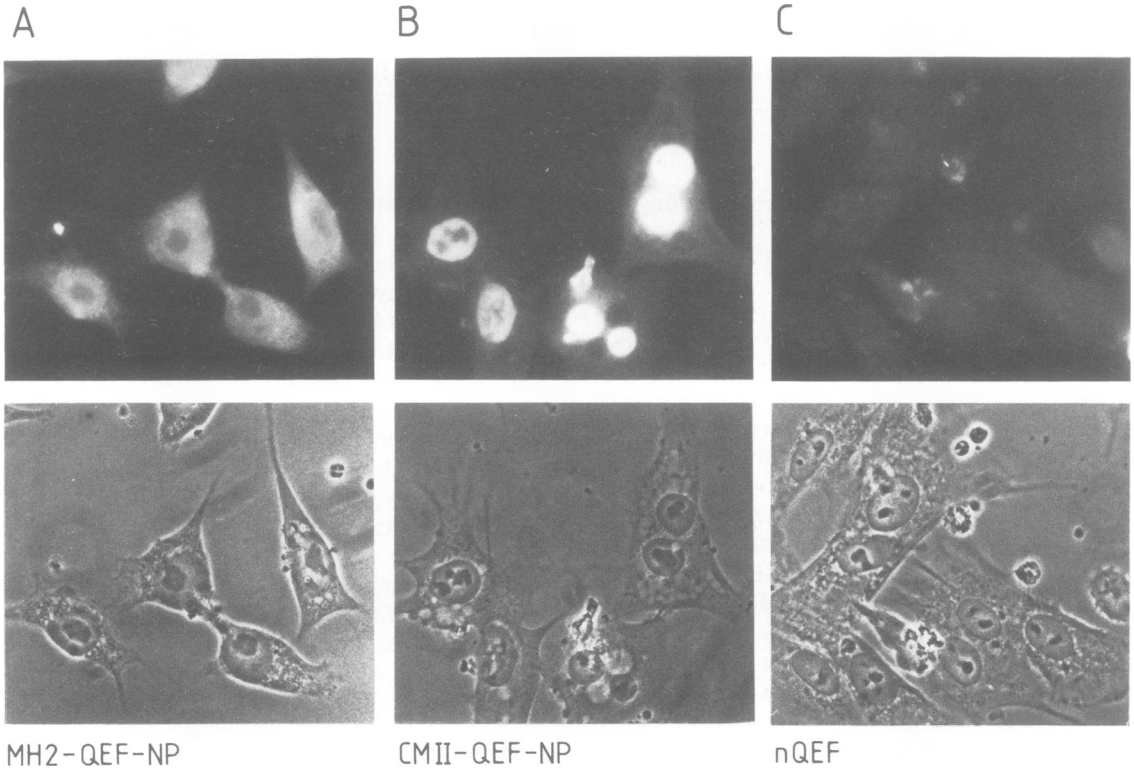

Fig. 1. Localization of gag-linked polyproteins by indirect immunofluorescence with monoclonal antibodies against p19 and fluorescein-labeled second antibody was performed as described (Donner *et al.*, 1982) with MH2-transformed non-producer quail fibroblasts (**A**), CMII-transformed non-producer quail fibroblasts (CMII-NP) (**B**) and normal quail cells (nQEF) (**C**). Phase-contrast pictures are shown for direct comparison. Magnification is 550 x.

tein. Figure 1 shows the result of this analysis. In contrast to the data described for MC29-transformed non-producer cells, the p100 from MH2-transformed fibroblasts was not located predominantly in the nucleus, but had a cytoplasmic distribution excluding nuclei and nucleoli. CMII-transformed quail fibroblasts, however, showed clear nuclear fluorescence similar to MC29-transformed cells (Figure 1A and B). Fluorescence of control cells was negative (Figure 1C). Phase contrast pictures from the identical cells are shown for comparison.

The fluorescence microscopy data were confirmed by fractionating radioactively labeled transformed cells into nuclear and cytoplasmic fractions as described (Bunte *et al.*, 1982). The distribution of p100 and p90 in the subcellular fractions was identified by indirect immunoprecipitations using an excess of antibody against p19. As a control of the quality of the cell fractionation, MH2-RAV60 cells were fractionated in parallel and the distribution of viral proteins such as $Pr180^{gag\text{-}pol}$, $Pr76^{gag}$ and p27 were determined. p27 is normally only present in the cytoplasmic fraction and Pr76 is also predominantly located in the cytoplasm, however, 10–20% of it was consistently found in the nucleus (Donner *et al.*, 1982; Enrietto and Erikson, 1982). The result of the fractionation is shown in Figure 2A and B. A quantitative evaluation of the precipitated proteins in the individual fractions is presented in Table I. The numbers correspond to the amounts of radioactively labeled proteins eluted from the gel and corrected for total volumes. The table confirmed the results obtained by immunofluorescence, the p100 protein of MH2 was predominantly cytoplasmic, whereas p90 of CMII resided predominantly in the nucleus and behaved very similarly to p110 of MC29.

Interaction of purified proteins with nucleic acids in vitro

[^{35}S]Methionine-labeled MH2- and CMII-transformed non-producer cells were lysed and applied to immunoaffinity columns containing monoclonal IgG directed against p19. The proteins were eluted using low pH buffer. Figure 3A and B show the ~2000-fold purified proteins eluted from the column, aliquots of which were directly applied to polyacrylamide gels. The purified polyproteins p100 and p90 were then analyzed for their capacities to bind to ^{3}H-labeled double-stranded quail fibroblast DNA in a filter-binding assay which was performed in the presence of 50 mM NaCl to reduce non-specific binding of protein to DNA. A similar experiment has already been described for p110 from MC29 (Donner *et al.*, 1983). Figure 4A shows the results of the filter-binding assay; p110 of MC29 is shown for comparison. All three proteins bound to DNA *in vitro* to similar degrees. No binding to DNA was found with purified p75, the gag-erbA protein from AEV-transformed cells (Moelling *et al.*, 1982; Donner *et al.*, 1983), and $Pr76^{gag}$ from SR-D virus-transformed cells (Donner *et al.*, 1982). Both results are shown in Figure 4A as negative controls.

Since a DNA-binding ability of the cytoplasmic MH2 polyprotein p100 was unexpected, other parameters were investigated to determine whether p100 exhibited properties correlating with its cytoplasmic localization. Such a difference was indeed detected when the purified p100 protein was analyzed for its ability to bind to single-stranded RNA *in vitro* in a filter-binding assay. The purified p100 of MH2 revealed a strong capacity to bind to RNA whereas all the other purified proteins, the CMII-specific p90 and the AEV-specific p75 and Pr76 from SR-D cells did not bind RNA

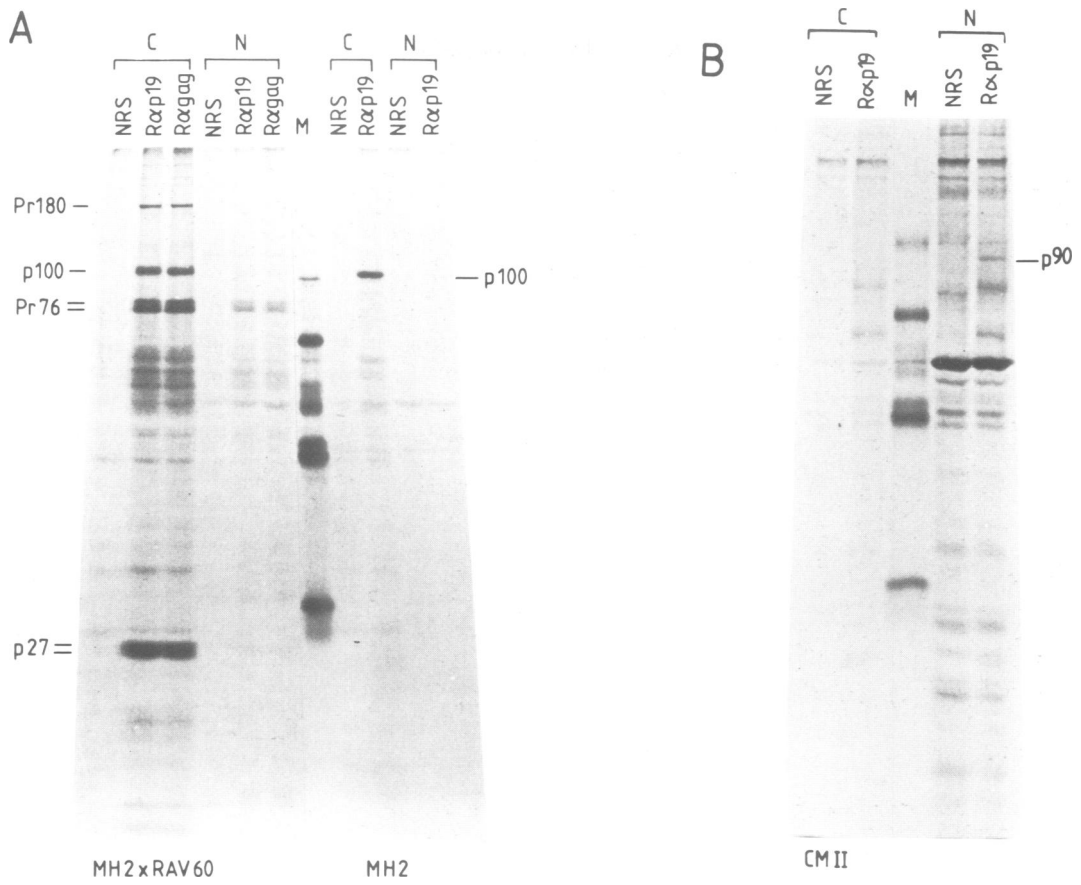

Fig. 2. Fractionation of MH2- and CMII-transformed fibroblasts. MH2 non-producer and MH2 x RAV60 superinfected quail cells (**A**) and CMII-non-producer cells (**B**) were labeled with [^{35}S]methionine for 2 h and were fractionated into a nuclear (N) and combined soluble fractions (C) as previously described (Bunte *et al.*, 1982). Standardized amounts of radioactive input (3 x 10^6 c.p.m.) corresponding to 10% of the nuclear and 2% of the soluble fractions were used for indirect immunoprecipitation using the following sera: NRS, normal rabbit serum; Rαp19, rabbit serum against p19; Rαgag, rabbit antiserum against all structural viral gag proteins. The precipitates were analyzed on polyacrylamide gels which were dried and exposed for autoradiography. p100 and p90 indicate the MH2-and CMII-transforming proteins, Pr180, Pr76 and p27 are gag proteins characteristic of the producer cells. M indicates ^{14}C-labeled mol. wt. markers (from top to bottom): 97 K, 68 K, 54 K, 44 K, 35 K.

Table I. Cellular distribution of various viral proteins

		MH2 x RAV60		MH2-NP		CMII-NP	
		S	N	S	N	S	N
Pr180$^{gag\text{-}pol}$	%	89	11	–	–	–	–
	c.p.m.	3680	640	–	–	–	–
p100(MH2)	%	91	9	93	7	–	–
	c.p.m.	10 000	900	10 110	837	–	–
p90(CMII)	%	–	–	–	–	29	71
	c.p.m.	–	–	–	–	1250	2992
Pr76^{gag}	%	86	15	–	–	–	–
	c.p.m.	13 250	2400	–	–	–	–
p27	%	93	7	–	–	–	–
	c.p.m.	19 270	1600	–	–	–	–
c.p.m. of the total lysate		1.1 x 10^8	1.9 x 10^7	1.6 x 10^8	3 x 10^7	1.8 x 10^8	4.0 x 10^7

[^{35}S]Methionine-labeled MH2-NP, MH2-RAV60 and CMII-NP cells (~10^7 cells each, labeled for 2 h with 250 μCi/ml of isotope) were fractionated into nuclear (N) and combined soluble fractions (S) as described (Bunte *et al.*, 1982). Standardized amounts of radioactive input of each fraction (3 x 10^6 c.p.m.) were used for immunoprecipitation. After gel electrophoresis and autoradiography of the precipitates, the amount of radioactivity of the various proteins was determined. The numbers indicated in the table were corrected for total volumes expressed as percentage (%) of the sum of S and N, and as c.p.m. of [^{35}S]methionine.

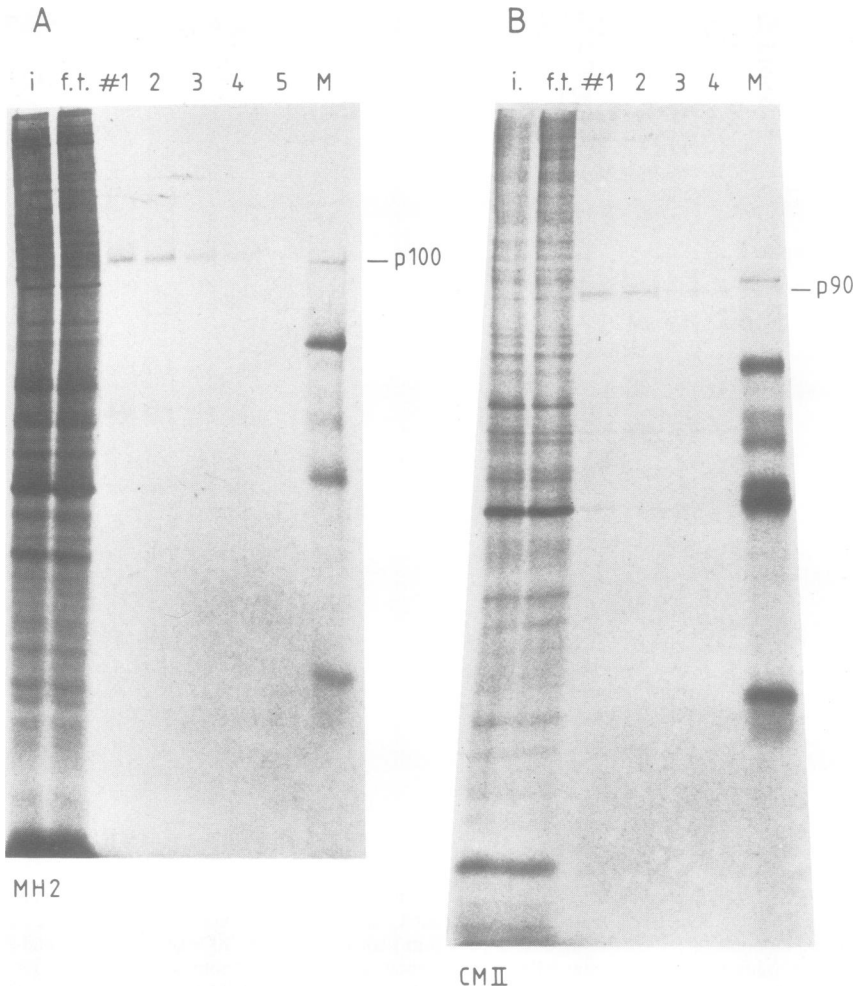

Fig. 3. Purification of MH2- and CMII-specific polyproteins. MH2 (**A**) and CMII (**B**) non-producer transformed fibroblasts (10^8 cells each) were labeled with [^{35}S]methionine for 2 h (250 μCi/ml), lysed and applied to immunoaffinity columns containing monoclonal IgG against p19. The transforming proteins were eluted with low pH buffer as described (Donner *et al.*, 1982). Aliquots of the input (i), flow-through (f.t.) material (corresponding to ~0.05% of the total each) and of the eluted fractions (# 1 through 4 and 5, respectively) (50 μl out of 1 ml fractions) were applied directly to 10% polyacrylamide gels and processed for autoradiography. M indicates mol. wt. markers (see legend to Figure 2).

under identical conditions (Figure 4B). The lack of binding by Pr76^{gag} was unexpected since the gag precursor contains p12, which has been shown to interact with DNA and RNA *in vitro* (Sykora and Moelling, 1982). However, Pr76 contains p12 as an integral part and this may mask its RNA binding ability. Furthermore, p12 binding studies were performed with larger amounts of proteins than were available to us. In the experiment shown in Figure 4B, poly(A)-containing total cellular RNA was used. The experiment was repeated with ^{32}P-labeled sucrose gradient-purified 28S rRNA with indistinguishable results (data not shown). 1 μg of p100 bound ~5 μg of RNA in both experiments. Binding to DNA was roughly of the same order of magnitude.

Since elution of proteins from the immunoaffinity columns involved a low pH buffer, an alternative approach was applied to prove a binding of the proteins in their native states to DNA and RNA. p100 was immobilized and purified on the immunoaffinity column without elution and was also found to bind to DNA and RNA (data not shown).

Since the properties of p100 differed from the gag-myc-type proteins p110 and p90, we considered the possibility that p100 was a gag-pol-related and not a gag-myc-type polyprotein. Previously published analyses of p100 did not rule out this possibility (Hu *et al.*, 1978; Hu and Vogt, 1979; Neil *et al.*, 1981). We therefore tested whether or not p100 was precipitated by antibodies against the viral RNA-dependent DNA polymerase. The result of an immunoprecipitation is shown in Figure 5 (left). p100 was not precipitated with the anti-polymerase serum used. The specificity of the serum for the polymerase was achieved by preabsorption of the serum with disrupted virus which is shown in Figure 5 (right) as a control. This experiment, however, does not rule out the possibility that only parts of the polymerase gene are fused to the *gag* gene which may be undetectable by the serum. Furthermore, we tested purified RNA-dependent DNA polymerase from virus particles (Moelling, 1974) for its ability to bind to RNA matching the amount of enzyme with the amount of p100 in a filter-binding assay. No binding at this concentration of enzyme was detectable (data not shown). Recently unpublished data were reported according to which no *pol* sequences were detectable using a pol-specific probe (Pachl *et al.*, 1983). We are presently investigating a possible biological function of the p100 protein by analyzing its influence on *in vitro* translation.

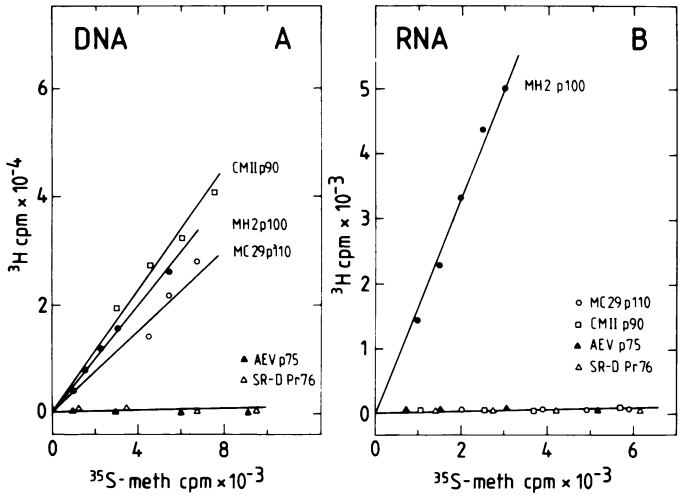

Fig. 4. Interaction of purified polyproteins with DNA (**A**) and RNA (**B**). (**A**) DNA filter-binding assays were performed in the presence of 50 mM NaCl as described in Materials and methods and Donner *et al.* (1982) with the following proteins purified by immunoaffinity column chromatography: p110 from MC29 (○——○), p100 from MH2 (●——●), p90 from CMII (□——□), p75 from AEV (▲——▲) and Pr76 from SR-D (△——△). Sheared ^{3}H-labeled double-stranded DNA from MC29-Q8-NP cells was used (1 μg corresponding to 1.3 x 10^{5} c.p.m. per assay). The amounts of proteins used per assay are indicated by the [^{35}S]methionine-labeled input, 5 x 10^{4} c.p.m. corresponding to ~1 μg of each of the purified proteins. (**B**) RNA fiter-binding assays were performed as described in Materials and methods. ^{3}H-labeled poly(A)-containing chicken cell RNA was used with 1 μg of RNA corresponding to 1.8 x 10^{4} c.p.m. per assay.

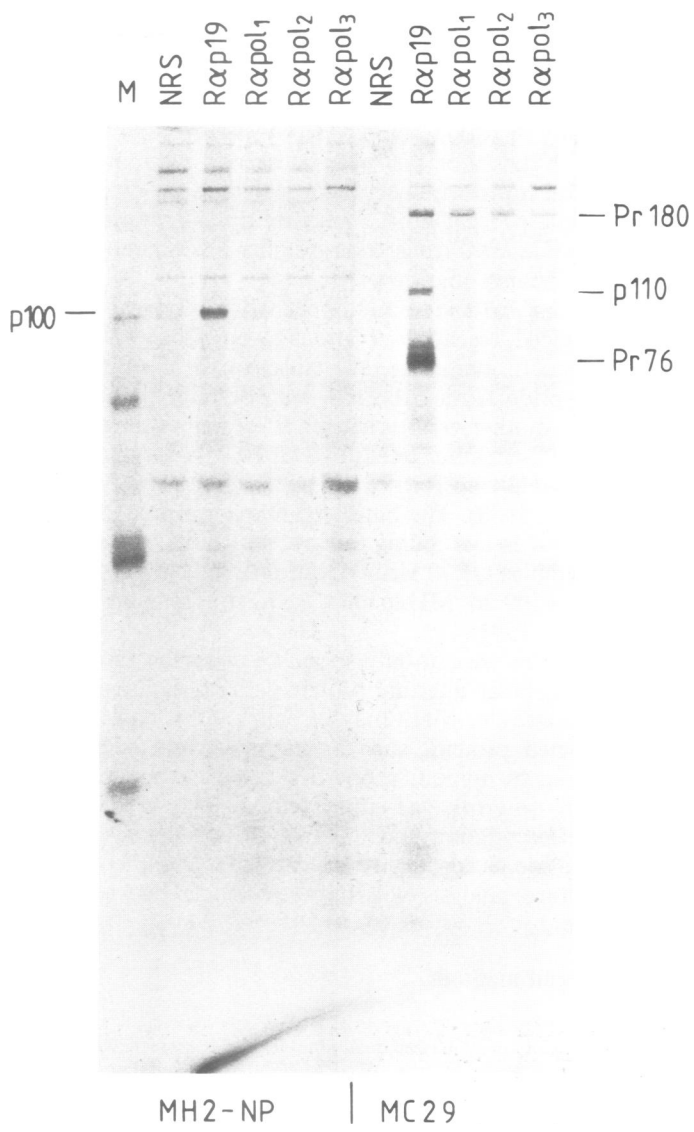

Fig. 5. Immunoprecipitation of p100 by antiserum against RNA-dependent DNA polymerase. [^{35}S]Methionine-labeled cellular extracts of MH2 non-producer (MH2-NP) and MC29 producer (MC29) cells were treated with various antisera for indirect immunoprecipitation. NRS, normal rabbit serum; Rαp19, rabbit antiserum against p19; Rαpol$_{1,2,3}$, rabbit antiserum against polymerase treated with 10, 20 and 30 μg of disrupted virus, respectively, for absorption of antibodies against viral structural proteins. Rαpol$_1$, e.g., no longer precipitates p100 (**left**), it precipitates Pr180$^{gag\text{-}pol}$ without precipitating Pr76 (**right**) indicating its pol-specificity. M represents ^{14}C-labeled mol. wt. markers (see legend to Figure 2).

Discussion

We have described previously some properties of the transforming protein gag-myc from MC29 acute avian leukemia viruses. As we and others have found, the transforming protein was located in the nucleus of transformed non-producer quail embryo fibroblasts (Donner *et al.*, 1982; Adams *et al.*, 1982). Furthermore, we demonstrated its close association with chromatin in transformed bone marrow cells and showed that it strongly bound to double-stranded DNA after extensive purification (Donner *et al.*, 1982; Bunte *et al.*, 1982). Since MC29 is a member of a group of viruses which comprises three other isolates, we investigated two of them, MH2 and CMII, of which transformed non-producer cell lines were established or available.

The transforming protein from CMII behaved indistinguishably from the MC29-tansforming protein in all parameters tested, as it was also located predominantly in the nucleus and bound to double-stranded DNA after purification. In fact, the biological properties described for CMII resemble those of MC29 most closely in that both viruses induce a similar spectrum of tumors and a similar pattern of target cell specificity (Linial, 1982).

The observations made with the MH2-transforming protein p100, however, were quite distinct from p110 of MC29. The gag-linked protein was clearly not located in the nucleus of transformed quail fibroblasts as shown by indirect immunofluorescence with monoclonal antibodies against p19 and cell fractionation. However, the purified protein interacted with double-stranded DNA *in vitro*. The biological significance of this DNA-protein interaction is not clear, and no parameters are known as yet to distinguish between the nature of this DNA binding and that of p110 of MC29 which correlates with transformation. DNA binding was reduced with proteins from transformation-defective mutants (Donner *et al.*, 1983). Mutants of MH2 are not available for a similar comparison.

The ability of p100 to bind to DNA in combination with its cytoplasmic localization raised the question of whether gag-myc proteins exhibit nuclear or cytoplasmic localizations dependent on the cell-cycle or growth conditions. A protein with such characteristics has been described. p53 is found in the nucleus of actively dividing cells and is cytoplasmic in confluent stationary cells (Dippold *et al.*, 1981). It is also associated with T-antigen of SV40-transformed cells (Lane and Crawford, 1979). We have therefore performed preliminary immunofluorescence analyses with MC29- and MH2-

transformed cells using monoclonal antibodies against p19 to investigate a cell-cycle-dependent localization of the polyproteins. No such variations could be detected (Greiser-Wilke, unpublished observation).

The ability of p100 to bind to RNA *in vitro* is a novel property which has not yet been observed with any other oncornaviral transforming protein including the cytoplasmic AEV-specific p75 gag-erbA. Whether this binding results in inhibition of *in vitro* translation is under investigation. Other functions are also unknown.

In summary, p100 does not appear to be a typical gag-myc protein. Indeed, recent observations by others lend support to this notion. In one case, the absence of a *myc*-specific phosphopeptide from p100 has been described (Ramsay *et al.*, 1982). Another very recent report describes the existence of MH2 p100-containing cells (MH2p100$^+$) as well as a very oncogenic MH2-transformed cell line lacking p100, MH2YS3 (Pachl *et al.*, 1983). The latter cell line appears to contain a subgenomic message coding for myc sequences – a situation which resembles OK10 virus (Graf and Stehelin, 1982). The nature of p100 in MH2p100$^+$ cells still remains unclear (Pachl *et al.*, 1983).

At this point we can only speculate upon the function of p100. It may be an aberrant protein with no significant function. Alternatively, MH2 may resemble AEV and code for two unrelated proteins such as gag-erbA and erbB. Even though transformation properties appear to reside predominantly on erbB, gag-erbA seems to play a role during cellular differentiation and to exert an enhancing effect on transformation according to recent data (Frykberg *et al.*, 1983). Future analysis will have to decide between these possibilities.

Materials and methods

Cells and viruses

MC29-Q8-NP is an established quail fibroblast cell line which does not produce any virus (Bister *et al.*, 1977). MH2-NP is a cell line of MH2-transformed quail non-producer fibroblasts which was kindly supplied to us by C. Moscovici, Gainsville, FL. MH2 x RAV60 cells were obtained by superinfection of the MH2 quail cell line with RAV60 helper virus. CMII-NP cells were established as a quail fibroblast cell line in this laboratory by standard procedures involving end point dilution (Vogt, 1969). The cells were grown in 1/2 Dulbecco's modified Eagle's medium (DMEM), 1/2 RPMI, 1 mM Hepes, 5% calf serum, 1% heat-inactivated (30 min 56°C) chicken serum and 0.5% dimethyl sulfoxide.

Cells were labeled with [^{35}S]methionine with 250–500 μCi/ml on 10 cm Petri dishes with cells at 70–80% confluency for 4 h.

Purification of all viral polyproteins was performed by immunoaffinity column chromatography using IgG of monoclonal antibodies against p19 as has been described (Greiser-Wilke *et al.*, 1981; Donner *et al.*, 1982).

Cellular fractionation of nuclei and soluble fractions, subsequent immunoprecipitation and quantitative evaluation of the radioactive material has been published before (Donner *et al.*, 1982; Bunte *et al.*, 1982).

Filter-binding assays were performed as described (Donner *et al.*, 1982) using a reaction mixture of a total volume of 500 μl containing 50 mM Tris-HCl, pH 8, 2 mM EDTA, 50 mM NaCl, 130 000 c.p.m. of ^{3}H-labeled double-stranded chicken DNA (sheared to ~10 kb), with a specific activity of 130 000 c.p.m./μg). The filter-binding assay using RNA was performed in the absence of NaCl using otherwise identical conditions. About 20 000 c.p.m. of ^{3}H-labelled poly(A)-containing cytoplsmic RNA with a specific activity of 20 000 c.p.m./μg were used.

Details of immunofluorescence analyses have been described previously (Donner *et al.*, 1982; Bunte *et al.*, 1982).

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