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Phosphorylation of specific sites in the *gag-myc* polyproteins encoded by MC29-type viruses correlates with their transforming ability

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The putative transforming proteins of the four acute leukaemia viruses belonging to the MC29 subgroup were shown to be phosphorylated *in vivo*. Comparison of the MC29 and CM11 encoded phosphoproteins revealed identical tryptic phosphopeptide maps, with both the *gag* and *myc* domains being phosphorylated. In contrast, the MH2 phosphoprotein was only phosphorylated on the *gag* domain. Analysis of partial transformation-defective MC29 deletion mutants revealed that the deletions had removed the *v-myc* specific phosphopeptides. Phosphoamino acid analysis showed that these deleted phosphopeptides were phosphorylated on threonine. Moreover, a back mutant that had regained transforming ability had regained these phosphopeptides. These studies correlate the phosphorylation of the *gag-myc* protein with the transformation capability of the virus.

Key words: phosphorylation/viral oncogenes/transformation-defective mutants

Introduction

The MC29 subgroup of defective leukaemia viruses (DLVs) contains four virus strains, MC29, CM11, MH2, and OK10. All share certain biological properties: *in vivo* they have a similar oncogenic spectrum causing myelocytomas, sarcomas, liver and kidney carcinomas, while *in vitro* they can transform fibroblasts and macrophage-like cells (for review see Graf and Beug, 1978). It has been proposed that these similarities in both pathogenicity and *in vitro* transformation potential are due to the presence of a MC29 subgroup-specific sequence (*v-myc*) derived from a cellular gene, *c-myc* (Bister and Duesberg, 1980; Bishop *et al.*, 1980; Stehelin *et al.*, 1980; Graf *et al.*, 1980). Since then studies using transformation-defective (*td*) mutants of MC29 have defined essential and non-essential regions of *v-myc*, the oncogene of MC29 (Ramsay *et al.*, 1980, 1982; Bister *et al.*, 1982; Enrietto and Hayman, 1982).

In MC29, CM11, and MH2 transformed non-producer cells the only viral gene products detected were polyproteins of 110 000 (p110), 90 000 (p90), and 100 000 (p100) daltons, respectively (Bister *et al.*, 1977, 1980a; Hayman *et al.*, 1979; Hu *et al.*, 1978). Tryptic peptide analysis of MC29 p110 and CM11 p90 showed that they contained two domains, one at the amino-terminal encoded by a partial *gag* gene and the other at the carboxy-terminal encoded by *v-myc* (Kitchener and Hayman, 1980). By analogy with MC29 and CM11, the MH2 p100 protein was considered to be a *gag-myc* polyprotein although this was not directly proven by the tryptic peptide analysis. Analysis of the OK10 virus genome and protein products demonstrated that it synthesised the *gag* gene precursor Pr76, and a 200 000 dalton polypeptide [p200 *gag, pol, myc* (Ramsay and Hayman, 1980; Bister *et al.*, 1980b)]. In addition, a subgenomic mRNA was detected in OK10 transformed cells which could code for a *v-myc* (Chiswell *et al.*, 1981). That these proteins have a role in transformation rests on three lines of evidence: (i) they are transformation specific, (ii) they are the only gene products so far detected in transformed cells, and (iii) *td* mutants of MC29 have genetic lesions that map to the *v-myc* domain of p110 (Ramsay *et al.*, 1982; Bister *et al.*, 1982; Enrietto and Hayman, 1982).

It has previously been observed that the MC29 p110 and CM11 p90 are phosphoproteins *in vivo* (Bister *et al.*, 1980a). Since phosphorylation has been widely recognised as a means for regulating the activity of proteins (Rubin and Rosen, 1975), determining the role of these post-translational modifications could increase our understanding of the control of *v-myc* polyproteins transforming function. In this study we begin such an analysis by determining the domain and specific sites on the *gag-myc* polyproteins that are phosphorylated and analyse the differences exhibited by two *td* mutants and one back-mutant of MC29.

Results

Phosphorylation of the gag gene related proteins of the MC29 subgroup of viruses

Previous analysis of the MC29 p110 protein and the CM11 p90 protein had shown that they were phosphorylated (Bister *et al.*, 1980a). Therefore, it was of interest to extend these studies to include the other two members of the MC29 subgroup of viruses, namely MH2 and OK10. Figure 1 shows that quail embryo fibroblasts transformed by MH2 (MH2AV) contain three *gag* gene related proteins, $Pr76^{gag}$, $Pr180^{gag\text{-}pol}$, and p100, as shown by labelling with [^{35}S]-methionine (Figure 1, track 1). By far the most heavily phosphorylated of these is the MH2 p100 protein (Figure 1, track 3). Similarly, although the OK10 p200 protein is a relatively minor virus-specific protein in OK10 transformed cells (Ramsay and Hayman, 1980) as shown (Figure 1, track 8), it is rather heavily phosphorylated (Figure 1, tracks 5 and 7). A comparison of the specific incorporation of [^{32}P]phosphate and [^{35}S]methionine into Pr76, MH2 p100, and OK10 p200 would suggest that the MH2 p100 and OK10 p200 proteins are more extensively phosphorylated at sites shared with Pr76, and/or that they contain specific phosphorylation sites not shared with Pr76.

Phosphorylation of the gag gene related proteins of partially transformation-defective MC29 mutants

In an attempt to determine whether phosphorylation is important for the function of these *gag-myc* proteins we decided to examine the phosphorylation of the proteins of three partially transformation-defective mutants of MC29, *td* 10A, *td* 10C and *td* 10H. These three viruses exhibit drastic reductions in their ability to transform bone marrow cells but are

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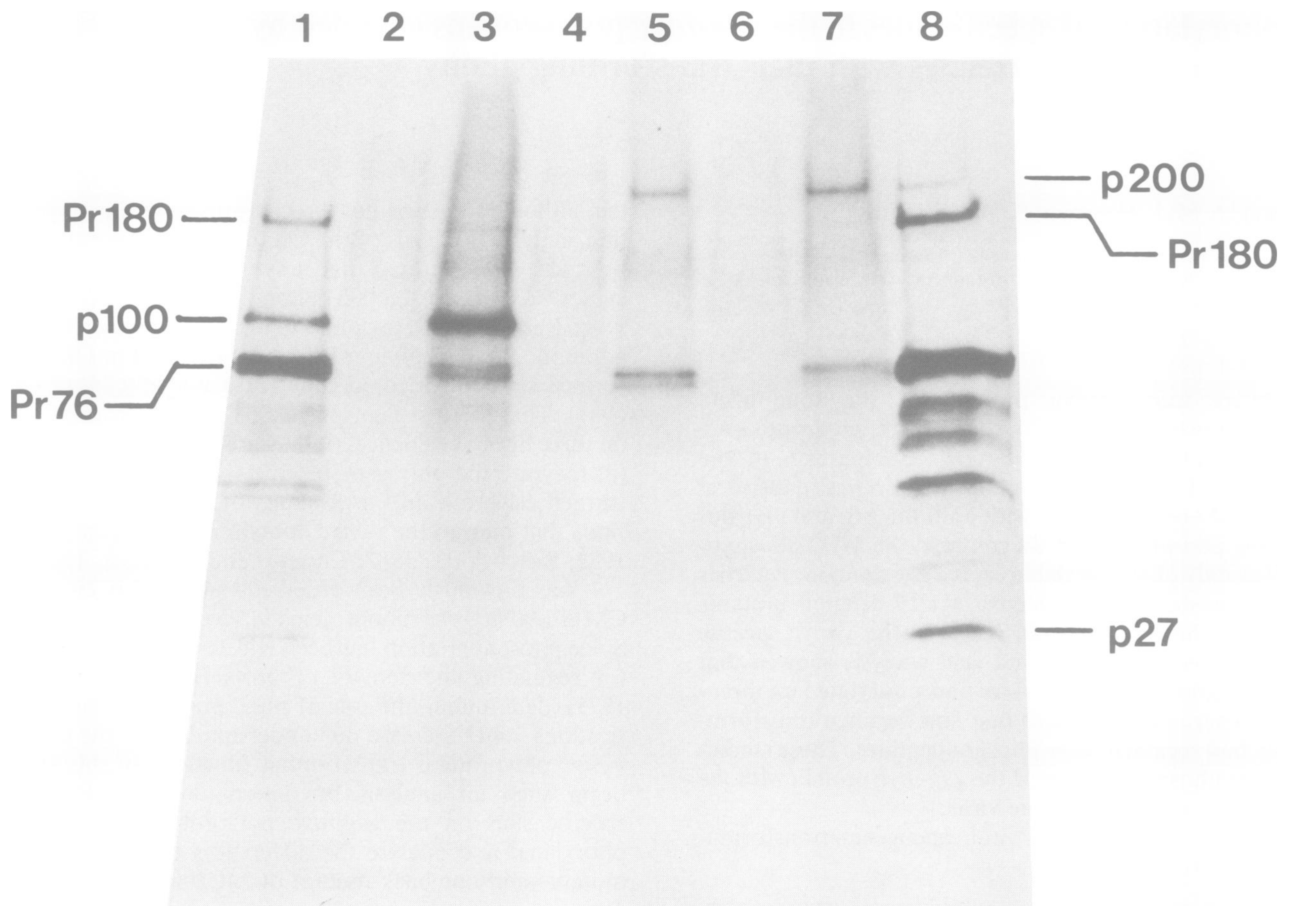

Fig. 1. Phosphorylation of non-structural, *gag* gene related proteins of acute leukemia viruses MH2 and OK10. Quail embryo fibroblasts transformed by MH2 (MH2 AV) (**lanes 1 – 3**), by OK10 (**lanes 4** and **5**), or by OK10 (RPV) (**lanes 6 – 8**) were labelled for 30 – 60 min with medium containing 80 μCi/ml [35S]methionine (**lanes 1** and **8**) or for 120 min with medium containing 500 μCi/ml H_3 $^{32}PO_4$ (**lanes 2 – 7**). Cellular detergent extracts were prepared and immunoprecipitations were carried out with rabbit preimmune serum (**lanes 2, 4,** and **6**) or with rabbit antiserum against *gag* proteins p27 and p19 (**lanes 1, 3, 5, 7, 8**) using equivalent numbers of TCA-precipitable counts. Electrophoresis in 6 – 18% gradient polyacrylamide-SDS gels and fluorography were carried out as described previously (Bister *et al.*, 1977).

still able to transform fibroblasts (Ramsay *et al.*, 1980). Figure 2A shows a comparison between the incorporation of [35S]methionine and [32P]phosphate into the *gag*-related proteins of these mutants and of wild-type (*wt*) MC29. The incorporation of [32P]phosphate relative to that of [35S]methionine is much lower in the *gag*-related proteins of all three *td* mutants as compared with the phosphorylation of the p110 protein of *wt* MC29 (Q8 line). A similar experiment was performed following superinfection of the non-producer cells with helper virus so that an internal comparison with the phosphorylation of Pr76*gag* could be carried out. Figure 2B confirms that the mutant proteins are far less phosphorylated than *wt* MC29 p110 (here the producer line Q10), and that their level of phosphorylation is comparable to that of the *gag* gene protein of the helper virus.

Localisation of the phosphorylation sites of MC29 p110, CM11 p90, and MH2 p100

The results shown in Figure 1 suggested that the *gag-myc* polyproteins were phosphorylated outside the *gag* domain of the molecule. To determine whether this was indeed the case the tryptic phosphopeptide maps of the MC29, CM11, and MH2 polyproteins were compared with that of the *gag* precursor polyprotein Pr76*gag*. The OK10-200K *gag-pol-myc* polyprotein was not included in this study as it proved impossible to isolate sufficient 32P-labelled polyprotein for analysis. Figure 3 shows that MC29-p110 (Figure 3A) and CM11-p90 (Figure 3B) give very similar maps, yield four major phosphopeptides labelled 1, 2, 3, and 4 and two other minor peptides 5 and 6 which, although always present, vary in intensity. The MC29 AV Pr76*gag* gave two phosphopeptides labelled x and y (Figure 3C) which when mixed with the phosphopeptides of p110 co-migrated with peptides 2 and 6 (Figure 3D). This suggests that the latter were the phosphopeptides from the *gag* domain of MC29 p110 and CM11 p90, whereas phosphopeptides 1, 3, and 4 are from the *v-myc* domain. However, it is possible that because of the nature of the hybrid protein, *gag* peptides might be phosphorylated in the *gag-myc* polyprotein but not in the *gag* gene product. In contrast the MH2 p100 yields a phosphopeptide map similar to that of Pr76*gag* suggesting that it was only phosphorylated on the *gag* domain (Figure 3E). This was a surprising result since if the MH2 p100 protein does contain *v-myc* encoded amino acid sequence, it might have been expected that the phosphorylation sites would have been conserved, as they have been between MC29 and CM11. However, it must be borne in mind that the *v-myc* sequences in MH2 only show ~60% homology with those of the other members of this group (Roussel *et al.*, 1979).

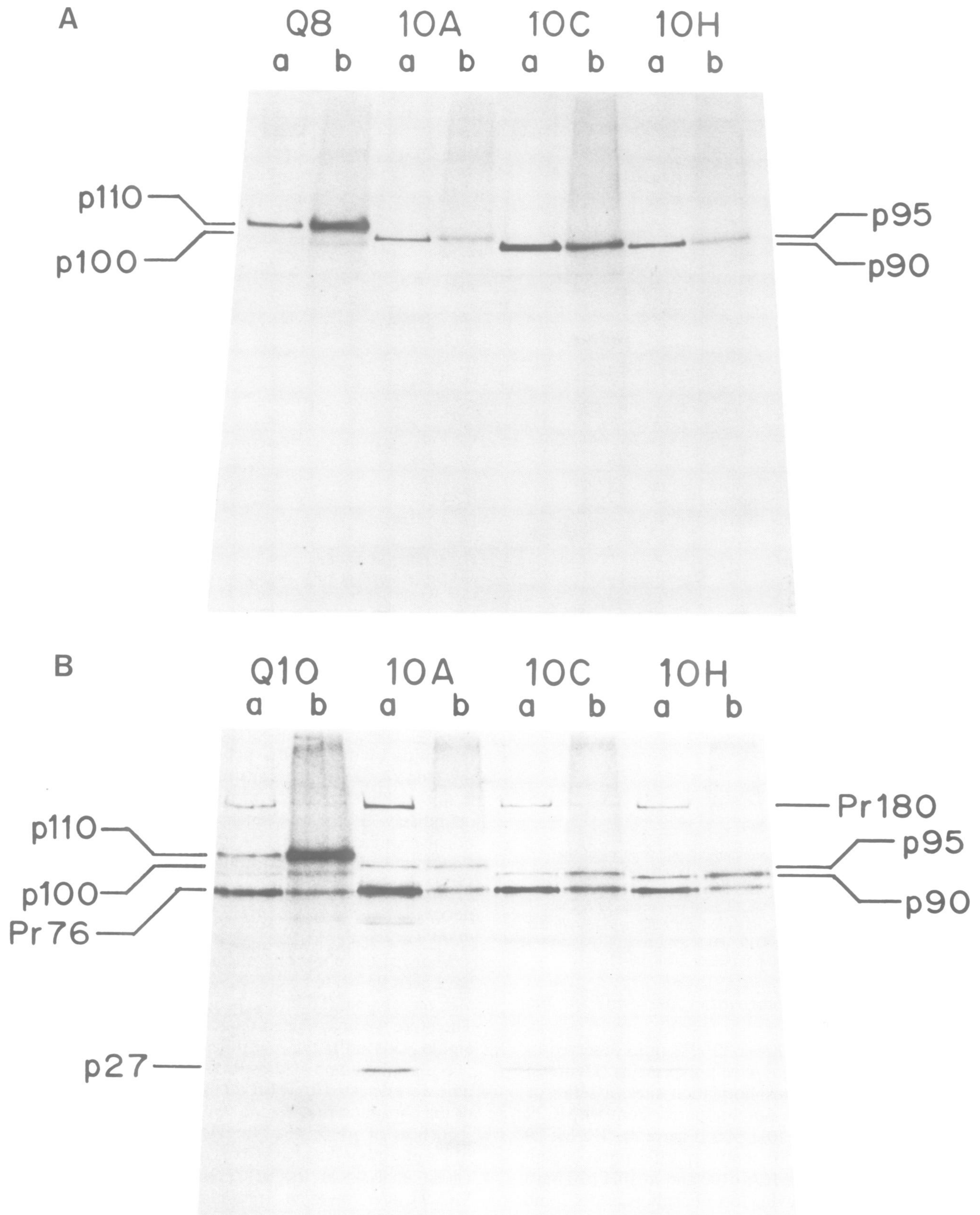

Fig. 2. Phosphorylation of non-structural, *gag* gene related proteins of *wt* and *td* MC29 viruses. (**A**) Non-producer cultures of quail embryo fibroblasts transformed by *wt* MC29, line Q8 (Bister *et al.*, 1977), or by *td* mutants 10A, 10C, and 10H (Ramsay *et al.*, 1980), were labelled for 120 min with medium containing 80 μCi/ml of [^{35}S]methionine (**a**) or 500 μCi/ml of $H_3\,^{32}PO_4$ (**b**). Cellular detergent extracts were prepared and immunoprecipitation of equivalent numbers of TCA precipitable counts was carried out with rabbit antiserum against whole disrupted Rous sarcoma virus. Electrophoresis in 6–18% gradient SDS-polyacrylamide gels and fluorography were carried out as described (Bister *et al.*, 1977). (**B**) Producer cultures of quail embryo fibroblasts transformed by *wt* MC29 (MCAV), line Q10 (Bister *et al.*, 1977), or by *td* mutants 10A, 10C, and 10H, pseudotyped with RPV, were labelled with [^{35}S]methionine (a) or $H_3\,^{32}PO_4$ (**b**) and analysed as in (**A**).

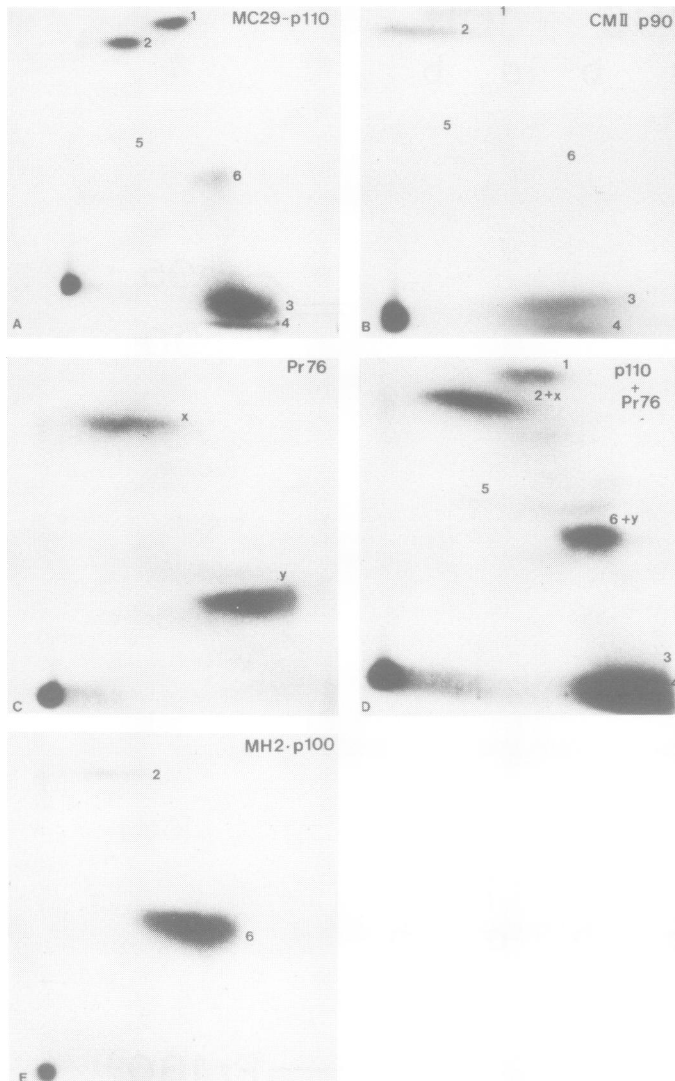

Fig. 3. Comparison of the phosphate-containing tryptic peptides of the *gag*-related proteins of MC29, CM11, and OK10. Tryptic peptide mapping of the ^{32}P-labelled proteins was as described in Materials and methods. The MC29-p110 (**A**) was isolated from the MC29 quail non-producer cell line Q8, the CM11-p90 (**B**) was from the CM11 producer cell line A4, Pr76 (**C**) was from the MC29 producer cell line Q10, (**D**) is a mixture of (**A**) and (**C**), and MH2-p100 was from MH2 non-producer quail cells.

Transformation-defective mutant viruses lose v-myc specific in vivo phosphorylation sites

To determine whether the lower ^{32}P/^{35}S ratio observed in the *td* MC29 virus encoded *gag-myc* polyproteins (Figure 2) resulted from the loss of phosphorylation sites, a series of tryptic phosphopeptide maps were prepared from *wt* MC29 p110, *td* 10A p100, and *td* 10H p90 (Figure 4). *Wt* MC29 p110 contains the three major *v-myc* specific phosphopeptides (1, 3, and 4) whereas *td* 10A p100 and *td* 10H p90 have only one (peptide 1), both having lost phosphopeptides 3 and 4. In addition, we prepared the phosphopeptide map of a recently isolated back-mutant from *td* 10H, called HB1, which has regained the *myc*-specific sequences lost from *td* 10H and now makes a p108 *gag-myc* protein (Ramsay *et al.*, 1982b). This reacquisition of *myc* sequences is accompanied by a restoration of the *in vitro* transforming activity to the virus. Interestingly, HB1 has regained phosphopeptides 3 and 4 (Figure 4B).

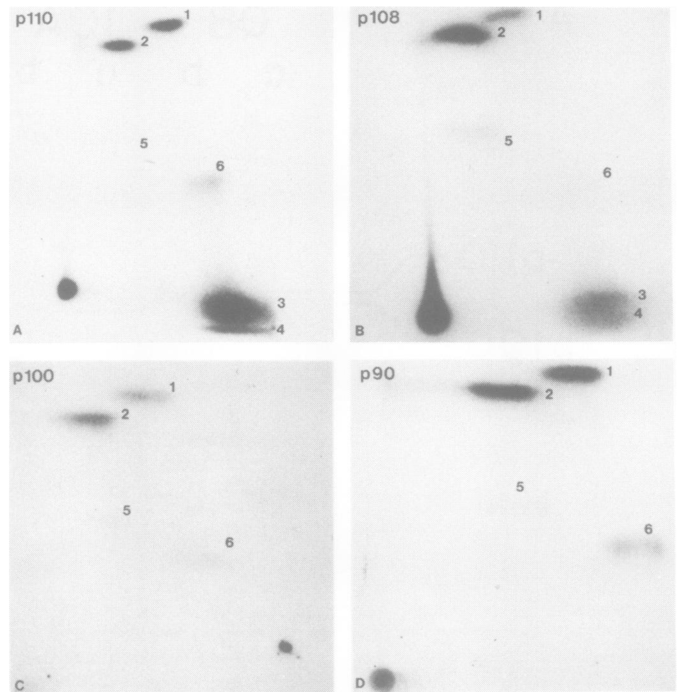

Fig. 4. Comparison of the phosphopeptide maps of the MC29 *td* mutants and the back mutant HB1. Tryptic peptide maps of the ^{32}P-labelled proteins were performed as described in Materials and methods. The proteins were all isolated from the appropriate nonproducer transformed quail fibroblasts.

This result correlates the loss of *v-myc* phosphopeptides with reduced transforming activity, while restoration in HB1 of these phosphopeptides accompanies regained transforming activity. These data thus implicate phosphorylation in transformation by the *gag-myc* polyprotein but do not show that this alone is vital to the transformation process. Moreover, it is not possible to assign these phosphorylation differences definitively to specific sequences in *v-myc* since conformational changes induced by deletion of sequences could influence the stability of phosphorylation at a distant site.

Identification of the phosphoamino-acids of the gag-myc polyproteins encoded by MH2, wt MC29, and the td MC29 mutants

Many of the phosphoproteins encoded by viral oncogenes have been shown to be phosphorylated not only on serine but also on tyrosine (Hunter and Sefton, 1980; Beemon, 1981). Therefore we investigated the identity of the phosphorylated amino acids in the *gag-myc* polyproteins. Figure 5 shows that two phosphoamino acids, phosphoserine and phosphothreonine were detected in *wt* MC29 p110 (Figure 5C and E). Some of the phosphoserine residues are likely to come from the p19 sequences of p110, since the *gag* gene-encoded polypeptide p19 is phosphorylated at serine residues (Erikson *et al.*, 1977). Direct analysis of the p19 precursor Pr76^{gag} encoded by MC29 AV revealed phosphoserine to be its only phosphoamino acid suggesting that the phosphothreonine was *v-myc* specific (Figure 5A). The *td* 10H p90 protein also contained only phosphoserine (Figure 5B); no phosphothreonine was detected whereas HB1 p108 contained both phosphoamino acids (Figure 5D). Thus, in MC29 the absence of phosphothreonine correlated with reduced transforming ability. However, the MH2 p100 polyprotein like Pr76^{gag} only contains phosphoserine, no phosphothreonine being

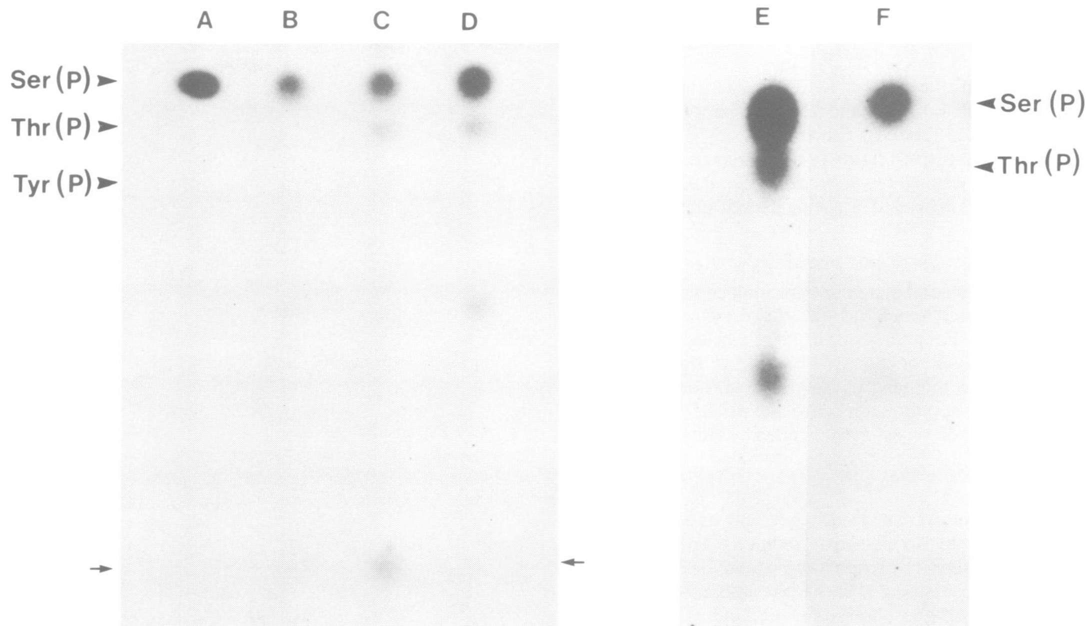

Fig. 5. Phosphoamino acid analysis. The analysis was performed as described in Materials and methods. The proteins are Pr76 (**A**), *td* 10H-p90 (**B**), MC29-p110 (**C** and **E**), HB1-p108 (**D**) and MH2-p100 (**F**). The origin is indicated by the arrow and the positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated.

detected (Figure 5F). This supports the results shown in Figure 3 which suggest that MH2 p100 only contains *gag* phosphopeptides.

Discussion

In this study we show that the putative transforming proteins of all MC29 subgroup viruses are phosphorylated *in vivo*. Since this modification may be essential for the transforming activity of these proteins we analysed several *gag-myc* phosphoproteins by tryptic peptide mapping and phosphoamino acid analysis. This strategy established three significant features of the MC29 and CM11 encoded phosphoproteins. (1) They yield identical phosphopeptide maps. (2) The *gag* and *v-myc* domains are phosphorylated. (3) Two phosphoamino acids are present; the *gag* domain contains phosphoserine whereas the *v-myc* domain contains phosphothreonine and possibly also phosphoserine. These results demonstrate a remarkable conservation of phosphorylation sites between the two proteins, implying functional significance. In contrast MH2 is heavily phosphorylated but only on the *gag* domain. There are two possible explanations for this difference between MC29 and CM11 on one hand and MH2 on the other. Since the MH2 *v-myc* sequence has significantly diverged from MC29 and CM11 (Roussel *et al.*, 1979), it is possible that *v-myc* specific phosphorylation sites were lost. Alternatively, the MH2 polyprotein may not contain *v-myc* encoded sequences. This possibility is supported by recent experiments which suggest that the *v-myc* information found in MH2 is expressed in a protein of 57 000 daltons synthesised from a subgenomic mRNA (Pachl *et al.*, 1982).

By comparing phosphopeptide maps of the *gag-myc* polyproteins encoded by *td* mutants of MC29 with that of the p110 protein encoded by *wt* MC29, it was shown that in the case of the *td* mutants the loss of specific phosphorylation sites in the *v-myc* domain of the *gag-myc* protein had occurred. The absence of these phosphorylation sites correlates with reduced transformation potential of these mutant proteins. The *td* viruses have, however, retained some oncogenic activity, in particular the transformation of cultured fibroblasts (Ramsay *et al.*, 1980). Hence the presence of these phosphorylation sites cannot be essential for all parameters of transformation. Phosphorylation may, however, modulate the transforming activity of the *gag-myc* polyprotein and hence perhaps also play a regulatory role in the function of the *c-myc* product. Evidence in support of this hypothesis comes from the HB1 virus generated from *td* 10H by presumed recombination with *c-myc* (Ramsay *et al.*, 1982b). The p108 protein it encodes has recovered the phosphorylation pattern characteristic of *wt* MC29 p110 suggesting that the *c-myc* gene product has conserved those phosphorylation sites which are encoded by *v-myc*.

Since the *gag*-related polyproteins of MC29 subgroup viruses have no autophosphorylating activity (Bister *et al.*, 1980a) it seems likely that cellular protein kinases must be responsible for the multiple *v-myc* specific phosphorylations. If these phosphorylation sites do serve a regulatory role then cellular protein kinases may also be able to control the activity of *gag-myc* polyproteins. This could explain, at least in part, the target cell specificity of transformation by MC29 in that only those cells able to carry out these phosphorylations would be susceptible to transformation by MC29.

Further investigations will be required before we can demonstrate a causal relationship between phosphorylation and the regulation of the biological activity of these proteins. Of particular interest in this regard is the recent observation that *wt* MC29 p110 is located predominantly in the nucleus of transformed cells where it specifically binds to chromatin (Donner *et al.*, 1982; Abrams *et al.*, 1982). This latter property could have a central role in the transforming activity of this polyprotein. Therefore, it will be of interest to determine whether the *v-myc* phosphorylation sites are involved in this DNA-protein interaction.

Materials and methods

Cells and viruses

Quail fibroblasts transformed by the different MC29-type viruses were used throughout this study and their isolation and characterisation has been reported previously; MC29 Q8 and Q10 cells (Bister *et al.*, 1977), CM11 A4 cells (Hayman *et al.*, 1979), OK10 B5 cells (Ramsay and Hayman, 1980), and MH-2 non-producer cells, which were kindly provided by Dr. P.K. Vogt (Hu *et al.*, 1978). The avian leukosis virus used was ring-necked pheasant virus (RPV).

Cell labelling and immunoprecipitation tryptic peptide mapping

Cells were labelled with [^{35}S]methionine, immunoprecipitated with anti-p27 antiserum and analysed by polyacrylamide gel electrophoresis as described previously (Hayman *et al.*, 1979). For analysis of proteins labelled with [^{32}P]-H_3PO_4, the procedure described by Bister *et al.* (1980) was used. Proteins were then analysed by tryptic peptide mapping as described in Kitchener and Hayman (1980), except that a 1% ammonium carbonate pH 8.9 buffer was used for electrophoresis, 55 min at 600 v, as recommended by Hunter and Sefton (1980).

Phosphoamino acid analysis

The ^{32}P-labelled phosphoproteins were analysed essentially as described by Hunter and Sefton (1980). The proteins were hydrolysed in 6 N HCl for 2 h at 110°C under N_2. The acid was removed by lyophilisation, and the hydrolysate dissolved in a marker mixture containing 0.01 M phosphoserine (Sigma), phosphothreonine (Sigma) and O^4-phosphotyrosine (a kind gift of A.E. Smith, N.I.M.R., London). The samples were spotted on t.l.c. plates and electrophoresed at pH 3.5 for 90 min at 600 v in acetic acid/pyridine/H_2O in the ratio 50:5:95. The phosphoamino acid markers were visualised by a 1% solution of ninhydrin in acetone, and the ^{32}P-labelled amino acids by autoradiography.

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