Detection and Localization of the v-myb(AMV) Gene Products of Avian Myeloblastosis Virus by a Synthetic Peptide Antiserum

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SUMMARY

An antiserum made against a synthetic peptide from an internal region of the predicted amino acid sequence of the avian myeloblastosis virus (AMV) transforming v-myb(AMV) gene identified two products, p46v-myb(AMV) and p32v-myb(AMV), which were localized in the nucleus of AMV-transformed myeloblasts. We propose that these proteins are the in vivo products of the v-myb(AMV) gene and thus the transforming protein(s) of AMV.

Avian myeloblastosis virus (AMV) is one of the defective leukaemia viruses (DLV), a subdivision of avian RNA tumour viruses (Weiss et al., 1982). It is distinguished from the other DLVs in that it can only transform haematopoietic cells. As with other DLVs, it has two components. One is the helper virus(es), the myeloblastosis-associated virus(es) [MAV-1 or -2, subgroup A or B, respectively (envelope antigenicity)]; the other is the transforming virus, AMV. It is defective due to replacement of the gene sequences necessary for replication, specifically the majority of the envelope gene, env, and the terminal portion of the polymerase gene, pol, with an oncogene, v-myb(AMV), which is presumed to be the source of the transforming protein of this virus. By in vitro translation, others (Anderson & Chen, 1981) and ourselves (Wright et al., 1984) identified at least two non-structural proteins which were thus potential products of the v-myb(AMV) gene. We (Wright et al., 1984) further showed that the peptides of the smaller [p34v-myb(AMV)] in vitro translation product were contained in those of the larger [p49v-myb(AMV)] product. We thus proposed that these two proteins were products of the v-myb(AMV) gene based on the predicted amino acid sequence (Klempnauer et al., 1982). This present report confirms and extends our in vitro studies by in vivo identification and localization of similar sized v-myb(AMV) products using a serum raised against a synthetic peptide from the predicted v-myb(AMV) sequence.

From the predicted v-myb(AMV) gene product sequence (Rushlow et al., 1982), we identified a synthetic peptide of predicted antigenicity (Hopp & Woods, 1981) (Fig. 1), which was synthesized by solid-phase peptide methodology (Barany & Merrifield, 1979), and purified by partition (LH-20) and gel filtration chromatography and shown to migrate as a single peak by reverse-phase chromatography and to contain the predicted ratio of amino acids. The synthetic peptide (Tyr-Thr-Asp-Glu-Asp-Pro-Glu-Lys-Glu-Arg-Ile-Lys-Glu [v-myb-(AMV)219-232], from residues 219 to 232 numbered assuming the six amino-terminal p19 gag residues joined to the v-myb(AMV) carboxy-terminal 382 residues (Klempnauer et al., 1982), was conjugated to bovine serum albumin (BSA) by the carbodiimide method (Goodfriend et al., 1964). We followed an immunization regimen reported (Wong & Goldberg, 1981) to produce antibody rapidly. After antibody was detected by immunoprecipitation
Cells (5 × 10⁷ to 10⁸) from a helper virus-free AMV-transformed myeloblast line (Moscovici, 1975) were labelled for 1-5 h in 1 ml RPMI 1640 medium minus cysteine (Gibco) containing 20 to 50 μCi/ml [³⁵S]cysteine (Amersham, 1090 to 1275 Ci/mmol). Other labelling protocols were used. These included doubling the labelling time, different medium (Joklik's modified MEM), a different amino acid [³⁵S]methionine (sp. act. similar to [³⁵S]cysteine), labelling with or without 5 % chicken serum plus 5 % calf serum, and with or without a 1 h preincubation without isotope in the labelling amino acid-free medium. No differences were seen among the different combinations. Labelled cells were lysed and fractionated into nuclear and cytoplasmic preparations as detailed (Neiman et al., 1980). The nuclear pellet was suspended in radioimmunoprecipitation (RIPA) buffer and the cytoplasmic supernatant was brought to the same concentration of RIPA buffer, immunoprecipitated and analysed by PAGE, as described (Wright et al., 1984), except that the Staphylococcus aureus was preincubated with macrophage lysate (macrophages being the normal cell counterpart of the myeloblasts; Moscovici, 1975).

Areas of polyacrylamide gel containing immunoprecipitated p46 and p32 from [³⁵S]cysteine-labelled cell extracts were cut from dried gels, prepared (Anderson & Chen, 1981) for in situ iodination with carrier-free ¹²⁵I (Amersham) (Elder et al., 1977) and digested in situ with 70% formic acid (cleavage at Asp-Pro) (Sonderegger et al., 1982) and analysed by PAGE.

The anti-synthetic peptide serum was used to identify and localize \( v-myb(AMV) \) gene products \textit{in vivo}. The serum immunoprecipitated a 46000 mol. wt. protein, p46 \textit{v-myb(AMV)}, which was localized to the nuclear preparation of AMV-transformed myeloblasts (Fig. 2, lane 4). The p46 \textit{v-myb(AMV)} was absent from the cytoplasmic preparation (Fig. 2, lane 2), and was not detected with normal serum (Fig. 2, lanes 1 and 3). The specificity of an anti-synthetic peptide serum was shown by preabsorbing it with the synthetic peptide, which reduced the immunoprecipitation (Fig. 3, lane 3) as compared to the unabsorbed antiserum (Fig. 3, lane 2). The pattern of the preimmune serum immunoprecipitation (lane 1) shows the non-specific bands. The serum preparations used in Fig. 3 were not preabsorbed with BSA, which may account for the background. In addition, the serum used in Fig. 3 was of a lower titre than that used in Fig. 2 and was used undiluted, whereas that used in Fig. 2 was diluted 1:10. Also, the cell lysate used in Fig. 3 was not ion-pair exchanged (Amons & Schrier, 1981) whereas that used in Fig. 2 was to remove
Fig. 2. SDS-PAGE analysis of immunoprecipitation of $[^{35}S]$cysteine-labelled myeloblast lysates, after removal of detergents by ion-pair exchange (Amons & Schrier, 1981). Serum diluted 1:10 in phosphate-buffered saline preabsorbed with 150 µg BSA per reaction (Wright et al., 1984). Lane 1, cytoplasm, normal serum; lane 2, cytoplasm, anti-synthetic peptide serum; lane 3, nucleus, normal serum; lane 4, nucleus, anti-synthetic peptide serum.

Fig. 3. SDS-PAGE analysis of immunoprecipitation of $[^{35}S]$cysteine-labelled myeloblast nuclear lysates. Lane 1, preimmune serum; lane 2, anti-synthetic peptide serum; lane 3, anti-synthetic peptide serum preincubated (Wright et al., 1984) with 10 µg synthetic peptide.

Fig. 4. SDS PAGE analysis of FA-digested $^{[35}S$]-labelled p32 (lane 1) and p46 (lane 2).

detergents to increase immunoprecipitation with anti-synthetic peptide sera (Mariottini et al., 1983). An additional band, of 32000 mol. wt., p32$^v$-myb(AMV), is seen in Fig. 3; this appears to be specific, since it is found only in precipitation by the non-preabsorbed anti-synthetic peptide sera (lane 2), and is not immunoprecipitated with preimmune (lane 1) or synthetic peptide-preabsorbed anti-synthetic peptide serum (lane 3). The lack of p32 in Fig. 2 is presumed to be due to inadequate protein (p46 is less intense than in Fig. 3). Both p46$^v$-myb(AMV) and p32$^v$-myb(AMV) were specifically immunoprecipitated by anti-synthetic peptide serum from $[^{35}S]$methionine-labelled myeloblast lysates in an identical pattern to the $[^{35}S]$cysteine-labelled cell lysate shown in Fig. 3.

To confirm the prediction that p32 was encoded by the $v$-myb(AMV) sequence, a formic acid (FA) digestion was performed on p32 and p46 and the products were compared (Fig. 4). The predicted FA peptides of these candidate $v$-myb(AMV) products are illustrated diagrammatically in Fig. 5. FA peptides of p46, as indicated in Fig. 4, conform to those predicted in Fig. 5. In lane 2 of Fig. 4, the FA digest of p46$^{v$-myb(AMV)} is shown. The p48 is assumed to be the full-length p46$^{v$-myb(AMV)}. The p29 is taken to be the predicted amino (26K) fragment and the p20 and/or p18 would be the predicted carboxy (18K) FA fragment of p46$^{v$-myb(AMV)}. The lower molecular weight
p15 may be explained by the spontaneous cleavage of p46\(^v\)-myb(AMV) to the possible carboxy p32 fragment (Fig. 5). The upper band of the p29 doublet in lane 2 of Fig. 4 is a candidate for the p32. The FA peptides of such a carboxy p32 fragment would be predicted to be 14K and 18K (Fig. 5). In lane 1 of Fig. 4, the FA digest of p32 is shown. The upper band is assumed to be residual undigested p32. The lower two bands best fit the predicted FA fragments of 14K and 18K (Fig. 5) of a p32 cleaved towards the carboxy end (Fig. 5). An amino-cleaved p32 is predicted to yield FA fragments of 26K and 6K (Fig. 5), and would thus not explain the p32 FA peptides observed in lane 1 of Fig. 4, without invoking further processing of the larger 26K predicted peptide (Fig. 5).

We have identified \(v\)-myb(AMV)-specific products of 46000 and 32000 mol. wt., which we term p46\(^v\)-myb(AMV) and p32\(^v\)-myb(AMV). We have also localized these AMV transforming gene proteins to the nucleus of AMV-transformed myeloblasts. While these studies were in progress, others (Boyle et al., 1983; Klempnauer et al., 1983) reported identification of \(v\)-myb(AMV)-specific proteins similar in size to the p46\(^v\)-myb(AMV) identified by us by using either anti-\(v\)-myb(AMV) synthetic peptides, different from ours, or antisera to a cloned, expressed partial \(v\)-myb(AMV) sequence. Nuclear localization of a similarly sized p46\(^v\)-myb(AMV) protein was also reported later (Boyle et al., 1984; Klempnauer et al., 1984). Detection of the smaller p32\(^v\)-myb(AMV) has not been previously reported. The immunizing reagents used by the two groups (Boyle et al., 1983; Klempnauer et al., 1983) overlap the \(v\)-myb(AMV) gene regions required to produce a protein having the size of p32\(^v\)-myb(AMV). Thus, we cannot explain why we, and not others, have detected p32\(^v\)-myb(AMV). The 32K protein could be a degradation product of p46\(^v\)-myb(AMV). We feel that its size, however, suggests that it might be an internally initiated product as we (Wright et al., 1984) have discussed previously for the p34\(^v\)-myb(AMV) in vitro translation product of AMV genomic RNA. From the FA digest data, the p32 is presumed to represent the carboxy two-thirds of p46\(^v\)-myb(AMV). The p32\(^v\)-myb(AMV) may be generated either from internal initiation at the first methionine of the \(v\)-myb(AMV) coding sequences or from proteolytic cleavage of p46\(^v\)-myb(AMV). The p46\(^v\)-myb(AMV) is the approximate molecular weight predicted by the nucleotide sequence (Klempnauer et al., 1982) and similar to that seen by us (Wright et al., 1984) on translation in vitro of AMV genomic RNA. Thus, we propose that the p32\(^v\)-myb(AMV) and p46\(^v\)-myb(AMV) proteins are the counterparts of those we generated by AMV genomic RNA translation in vitro.

The nuclear location of the \(v\)-myb(AMV) products is not unexpected, since the \(v\)-myb(AMV) gene has stretches enriched in the basic amino acids, lysine and arginine, similar to certain histones and the predicted myc gene product (Alitalo et al., 1983), which is located in the nucleus.
(Donner et al., 1982; Abrams et al., 1982). Thus, it might be speculated that the v-myb(AMV) products may bind to DNA and may transform a cell through a mechanism similar to that of other nuclear-located transforming proteins.

We have searched for but not detected a v-myb(AMV)-specific product in the myeloblast normal cell counterpart, the macrophage. Thus, we cannot address the differences between the sizes of reported (Boyle et al., 1983; Klempnauer et al., 1983) v-myb(AMV)-specific proteins in different non-myeloblast cells, although one (Boyle et al., 1983) was from normal thymus cells while the other (Klempnauer et al., 1983) was from a non-myeloblast transformed cell.

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