MOLECULAR AND CELLULAR BIOLOGY, Dec. 1984, p. 2843–2850
0270-7306/84/122843-08$02.00/0

Vol. 4, No. 12

Isolation of Monoclonal Antibodies Specific for Products of Avian Oncogene *myb*

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Received 29 June 1984/Accepted 21 August 1984

We isolated a series of monoclonal antibodies which were raised against a bacterially expressed protein, bp37$^{v\text{-}myb}$, and coded for by part of the avian v-*myb* gene. These monoclonal antibodies recognized a range of antigenic specificities on bp37$^{v\text{-}myb}$, and this was reflected in their differing specificities for the gene products of the v-*myb*, c-*myb*, and E26 viral oncogenes. One monoclonal antibody recognized, in addition to the v-*myb* and c-*myb* gene products, a conserved nuclear protein found in all tested cells. We describe the characterization of these monoclonal antibodies.

Avian myeloblastosis virus (AMV) is a replication-defective avian retrovirus which causes acute myeloblastic leukemia in chickens and also transforms myelomonocytic cells in vitro (15). The genome of AMV contains the oncogene v-*myb* which is responsible for the oncogenicity of the virus and which codes for a 45,000-dalton protein, p45$^{v\text{-}myb}$ (10). Like most of the other known retroviral oncogenes, v-*myb* appears to have arisen by viral transduction of an evolutionarily conserved normal cellular gene, the proto-oncogene c-*myb*. The functions of both v-*myb* and c-*myb* are at present obscure, but it is thought that the *myb* gene products possess none of the protein kinase activity demonstrated by certain other oncogene products.

A major requirement in the study of the role of v-*myb* and c-*myb* in transformed and normal cells is for a specific antiserum to their respective gene products. However, classical tumor regressor sera obtained from chickens bearing AMV-induced leukemias have not proved effective in this regard. Instead, anti-*myb* sera have been prepared against a bacterially expressed fusion protein (bp37$^{v\text{-}myb}$) which contains a large portion of the p45$^{v\text{-}myb}$ sequence fused to the 22 N-terminal amino acids of human growth hormone (10). Using an alternative approach, Boyle et al. (1) raised antisera against synthetic peptides whose sequences are determined by the nucleic acid sequence of v-*myb*. These antisera have proved useful in defining the protein products of both v-*myb* and c-*myb* genes.

To provide a permanent supply of specific, homogenous, and high-titer antibodies specific for the *myb* gene products, we made monoclonal antibodies (MAbs) from mice that were immunized with the bacterially expressed fusion protein bp37$^{v\text{-}myb}$. We generated a number of hybridoma proteins which recognized various antigenic sites on bp37$^{v\text{-}myb}$ and cross-reacted with the native gene products of AMV v-*myb*, c-*myb*, and E26 v-*myb* genes. One MAb recognized a highly conserved normal cellular protein in addition to v-*myb* and c-*myb* gene products. Here we describe the characterization of some of these MAbs.

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MATERIALS AND METHODS

Cell lines used. The AMV-transformed myeloblast line BM-2 (16), the E26 virus-transformed myeloblast line PJ212 (C. Moscovici, personal communication), the avian leukosis virus-transformed lymphoma line R2B (2), avian erythroblastosis virus-transformed erythroblasts, MC29 virus-transformed quail fibroblasts, and normal primary chicken fibroblasts were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 10% tryptone phosphate broth, 1% chicken serum, 5 mM glutamine, and 100 μg each of penicillin and streptomycin per ml. Hybridoma lines were cultured in the same medium lacking chicken serum and tryptone phosphate broth. In addition, hybridoma medium contained 5×10^{-5} M 2-mercaptoethanol.

v-*myb* expression vector and production and purification of bp37$^{v\text{-}myb}$. The v-*myb* expression vector pHGH*myb*M2 has been described previously (10). *Escherichia coli* 294 cells carrying the vector were grown and induced, and the insoluble material from the bacterial lysate was prepared as before (10). The further purification of bp37$^{v\text{-}myb}$ will be described elsewhere (G. Evan, J. Immunol. Methods, in press). Briefly, 1 to 2 mg of partially purified bp37$^{v\text{-}myb}$ was dissolved in 2% sodium dodecyl sulfate (SDS)–125 mM Tris-hydrochloride (pH 6.8)–10% 2-mercaptoethanol–8 M urea and fractionated on a preparative 12.5% SDS-polyacrylamide gel. Protein was identified by staining the gel with 0.25 M KCl (8), and the major band, containing bp37$^{v\text{-}myb}$, was excised. The gel slice was briefly homogenized, and the protein was eluted overnight into phosphate-buffered saline (PBS)–0.1% SDS with occasional shaking.

For use in an enzyme-linked immunosorbent assay (ELISA), the bp37$^{v\text{-}myb}$ eluted from the gel was clarified by centrifugation at 10,000 × *g* for 10 min, adjusted to a protein concentration of 0.05 to 0.1 mg/ml in PBS–0.1% SDS, and aliquoted into glutaraldehyde-activated microtiter plates (Evan, in press).

Immunization, fusion, and screening of hybridoma culture supernatants. Six mice each of the strains BALB/c, C3H, SJL, C57B16, and BALB/c × B6 F_1 were tested for immune response to bp37$^{v\text{-}myb}$. Unlike responses to many other bacterially expressed fusion proteins (unpublished data), there was no observable strain specificity in immune response to bp37$^{v\text{-}myb}$. All mice responded to bp37$^{v\text{-}myb}$ as judged by immunoblotting assays and ELISA. For the fusion

described in this paper, six BALB/c female mice were immunized with 50 μg of Nonidet P-40 (NP-40)-insoluble extracts of bacterial lysate (estimated at ca. 60% bp37$^{v\text{-}myb}$) in Freund complete adjuvant in the hind footpads. Two and 4 weeks later, mice were re-immunized intraperitoneally with an additional 50 μg of extract as a suspension in 0.9% NaCl. Before the fusion, the mouse showing the highest anti-bp37$^{v\text{-}myb}$ titer was immunized intraperitoneally as before. Six days later, the animal was sacrificed, its spleen was removed and disaggregated, and the splenocytes were fused to SP2 myeloma cells by the method of Fazekas de St. Groth and Scheidegger (6). Hybrid cells were immediately dispersed into 960 wells in hypoxanthine-aminopterin-thymidine medium over a layer of peritoneal exudate feeder cells. Upon reaching confluence, each culture supernatant was assayed by ELISA for activity against bp37$^{v\text{-}myb}$ and the components of a control bacterial extract. Positive supernatants were further assayed on Western blots of bp37$^{v\text{-}myb}$ fractionated on SDS-polyacrylamide gels. Cells from wells positive for anti-bp37$^{v\text{-}myb}$ activity by both ELISA and immunoblotting were cloned by limiting dilution.

ELISA. ELISA was performed as described by Evan (in press). Briefly, flexible polyvinyl chloride 96-well microtiter plates were activated by incubation with 0.25% glutaraldehyde in PBS and then coated with purified bp37$^{v\text{-}myb}$ dissolved in PBS–0.1% SDS. After antigen adsorption, microtiter wells were washed three times in Tris-buffered saline (TBS; 50 mM Tris-hydrochloride, 144 mM NaCl [pH 8.2]) and blocked in TBS containing 10% fetal calf serum. Plates were then stored at −20°C. For ELISA, 100 μl of appropriately diluted antiserum or culture supernatant was added to each well and incubated at room temperature for 30 min. Plates were washed three times by immersion in TBS followed by shaking dry, and 100 μl of protein A–β-galactosidase conjugate (Boeringher Mannheim Ltd., diluted 1:300 in TBS–10% fetal calf serum before use) was then added to each well. After a 30-min incubation at room temperature, plates were washed three times as before, and 100 μl of substrate solution (4 mg of *o*-nitrophenyl-β-D-galactopyranoside per ml in TBS–5 mM $MgCl_2$–100 mM 2-mercaptoethanol) was added to each well. For screening fusions, plates were read by eye after 45 min at room temperature. For other purposes (e.g., titrations), the optical density at 406 nm of wells was read with a Titertek Multiscan ELISA plate reader.

Western blot immunoassays. Samples run on 1.5-mm-thick SDS-polyacrylamide gels were electrophoretically transferred onto nitrocellulose (Schleicher and Schuell Ltd.) at 50 V for 4 h in 0.19 M glycine–20 mM Trisma base–20% methanol with a Bio-Rad Trans-Blot cell. The nitrocellulose blot was washed for 5 min in TBS, wrapped in Saran Wrap and stored at −20°C until used. There was no deterioration of the assay observed, even after blots had been stored for as long as 1 year.

To assay culture supernatants on blots of *E. coli* extracts containing bp37$^{v\text{-}myb}$, blots were thawed and cut into longitudinal strips; the strips were assayed by the method of Towbin et al. (21), except that protein A-horseradish peroxidase (HRP) conjugate was used at 3 μg/ml instead of HRP-conjugated anti-mouse antibody. 4-Chloronaphthol (4 mg/ml dissolved in TBS containing 20% methanol and 1 μl of H_2O_2 per ml) was used as the chromogenic substrate.

For assaying blots of cell lysates, blots were incubated for 2 h with primary antibody and washed once in TBS, once in TBS containing 0.1% NP-40, and once again in TBS. Each wash was for 5 min. Blots were then incubated for 1 h in rabbit anti-mouse immunoglobulin-HRP or swine anti-rabbit immunoglobulin-HRP (Dako, Ltd.) as appropriate, washed as before, and then incubated for 20 min in 3 μg of protein A-HRP per ml. After washing again, filters were incubated in TBS containing 0.5 mg of diaminobenzidene per ml, 0.04% $NiCl_2$, and 0.2 μl of 30% H_2O_2 per ml. The reaction was terminated by transferring filters to TBS containing 0.1% sodium azide, and the filters were then dried in the dark. All antisera and protein A conjugates were diluted in TBS–10% FCS.

Purification of MAbs. MAbs used for immunoprecipitation studies in this report were all purified by protein A-Sepharose affinity chromatography from overgrown culture supernatants. Culture supernatant was passed through a 4-ml protein A-Sepharose column at pH 8.2. The column was washed with TBS, and MAb was eluted with 3 column volumes of 0.1 M sodium citrate (pH 2.8). The eluate was neutralized with 1 M NaOH, and MAb was precipitated by the addition of 1 volume of saturated ammonium sulphate solution. The precipitate was collected by centrifugation, drained, dissolved in 1 ml of deionized water, and dialyzed against PBS–0.1% sodium azide. Before use, the antibody solution was centrifuged to pellet debris. For long-term storage, purified MAb was stored in PBS–0.1% sodium azide–50% glycerol at −20°C.

Radiolabeling of cells. Cells to be labeled with [^{35}S]methionine were suspended for 30 min at 37°C at 1×10^6 to 5×10^6 cells per ml in methionine-free RPMI 1640 containing appropriate dialyzed sera. [^{35}S]methionine (Amersham; 600 Ci/mmol) was then added to a final concentration of 0.1 to 1 mCi/ml, and the cells were incubated for 1 to 2 h at 37°C. After labeling, cells were washed three times in ice-cold PBS containing 0.1% sodium azide, pelleted, and taken up in lysis buffer (see below).

Immunoprecipitation. Immunoprecipitation was performed as described previously (4), but with some modifications. Namely, cells were lysed in 0.5% NP-40–0.5% sodium deoxycholate–0.1% SDS–2% aprotinin (Sigma)–25 mM Tris-hydrochloride–144 mM NaCl (pH 8.2), and lysates were sheared through a 25-gauge needle until no longer viscous before clarifying by centrifugation at $10,000 \times g$ for 20 min. Also, the wash buffer used was TBS (pH 8.2) containing 0.5% NP-40–0.5% sodium deoxycholate–0.1% SDS.

Approximately equivalent amounts of the various monoclonal antibodies purified with protein A-Sepharose were used for each immunoprecipitation. In addition, the amount of antibody used for each immunoprecipitation was estimated by staining the SDS-polyacrylamide gel of fractionated immunoprecipitates with Coomassie brilliant blue before fluorography.

SDS-polyacrylamide gel electrophoresis and V8 peptide mapping. SDS-polyacrylamide gels were run as described by Laemmli (11). Peptide maps with staphylococcal V8 protease were performed by the method of Cleveland et al. (3).

RESULTS

Immunizations, fusions, and screening. Mice of all strains tested responded immunologically to bp37$^{v\text{-}myb}$ whether the immunogen was administered in purified form or as a partially pure, detergent-insoluble extract of bacterial lysate. In the latter case, however, a significant immune response was observed to a bacterial component which was also present in the partially purified extract from control bacteria not carrying the pHGH*myb*M2 plasmid (Evan, in press). By measuring the daily rise in anti-bp37$^{v\text{-}myb}$ titer after intraperitoneal administration of *myb* extract to hyperimmune mice, 6 days

TABLE 1. Basic features of Myb2 MAbs

Monoclonal antibody	Subtype	Protein A binding	Ascites fluid titer (1/2 maximal binding)
Myb2-2	IgG2a · K	+	1:16,000
Myb2-3	IgG2b · K	+	1:4,000
Myb2-6	IgG2a · K	+	1:2,000
Myb2-7	IgG1 · K	+	1:4,000
Myb2-10	IgG3 · K	+	ND[a]
Myb2-32	IgG2a · K	+	1:250
Myb2-36	IgG2a · K	+	ND
Myb2-37	IgG2b · K	+	1:8,000

[a] ND, Not determined.

post-immunization was estimated as the optimal time for fusions. Accordingly, the BALB/c mouse determined to have the best immune response to bp37$^{v\text{-}myb}$ was sacrificed 6 days after an intraperitoneal boost immunization. After fusion, hybrid cells were divided among 10 96-well microtiter plates. Thirty-five supernatants were scored positive against bp37$^{v\text{-}myb}$ (17 strong, 18 weak), 28 were scored positive on plates adsorbed with extract from control bacteria, and 1 was positive on both test and control plates. Of the 17 strong positives, 11 survived 2 weeks of additional growth. Of those 11, 9 were still positive after cloning. MAbs Myb2-2, Myb2-3, Myb2-6, Myb2-7, Myb2-10, Myb2-32, Myb2-36, and Myb2-37 were selected for further study (Table 1). All of the Myb2 MAbs further described in this paper bound bp37$^{v\text{-}myb}$ when assayed on immunoblots of SDS-polyacrylamide gels of bacterial lysates containing bp37$^{v\text{-}myb}$.

MAbs. The MAbs described here were all selected for protein A binding at pH 8.2. At this pH, immunoglobulin G of most subclasses binds protein A (5), and this was demonstrated by the range of immunoglobulin G subclasses in Table 1. In addition, high titers of anti-bp37$^{v\text{-}myb}$ activity were observed for ascites fluids obtained from BALB/c mice bearing most of the hybridomas (Table 1), but these ascites fluids were not used in this report.

In this study, MAbs used for immunoadsorption experiments were purified from 1 to 2 liters of overgrown culture supernatants by affinity chromatography on protein A-Sepharose. Were we to assume 100% yields by this technique, hybridoma protein concentrations in culture supernatants could be estimated to be in the range of 20 to 60 μg/ml for each of the hybridoma lines assayed.

Antigenic determinants recognized on bp37$^{v\text{-}myb}$. The bacterial immunogen bp37$^{v\text{-}myb}$ used in this study is a fragment of the native v-*myb* gene product. Moreover, the bp37$^{v\text{-}myb}$ target in all screening assays was at least partially denatured. We therefore consider it likely that the determinants recognized by the Myb2 MAbs are either primary sequences or possibly highly stable secondary structures rather than the kinds of epitopes formed by tertiary structure which are present in most native proteins. The Myb2 MAbs recognize bp37$^{v\text{-}myb}$ on immunoblots. If the Myb2 MAbs do indeed recognize primary sequence epitopes, a concommitant prediction might therefore be that they also recognize peptide fragments of the bp37$^{v\text{-}myb}$ protein on immunoblots. To test this, purified bp37$^{v\text{-}myb}$ was digested with V8 protease by the method of Cleveland et al. (3), and the peptides were electroblotted onto nitrocellulose. The blot was then probed with each of the Myb2 MAbs (Fig. 1) as well as with antiserum from the mouse from which the MAbs were derived. All of the MAbs recognized submolecular V8 peptide fragments of bp37$^{v\text{-}myb}$ (Fig. 1). V8 digestion of protein bands in SDS-polyacrylamide gels was almost always partial under the conditions we routinely used for Cleveland peptide mapping. Therefore, we were not surprised to see that most MAbs recognized several digest fragments rather than one alone. The result of this experiment does, however, give us an immunopeptide map which allows us to segregate at least three MAbs (Myb2-2, Myb2-3, and Myb2-32) into a common class.

To gain further information about the topography of binding sites recognized by the Myb2 MAbs, ^{3}H-labeled hybridoma proteins were purified. Excess amounts of each unlabeled MAb were then assayed for their ability to block ^{3}H-MAb binding to bp37$^{v\text{-}myb}$ adsorbed to microtiter plates. The results of this study (Table 2) were in good agreement with the immunopeptide map data (see above). Myb2-2, Myb2-3, and Myb2-32 mutually blocked the binding of each other and thus appeared to bind at, or close to, the same site on bp37$^{v\text{-}myb}$. In addition, Myb2-6 and Myb2-37 partially

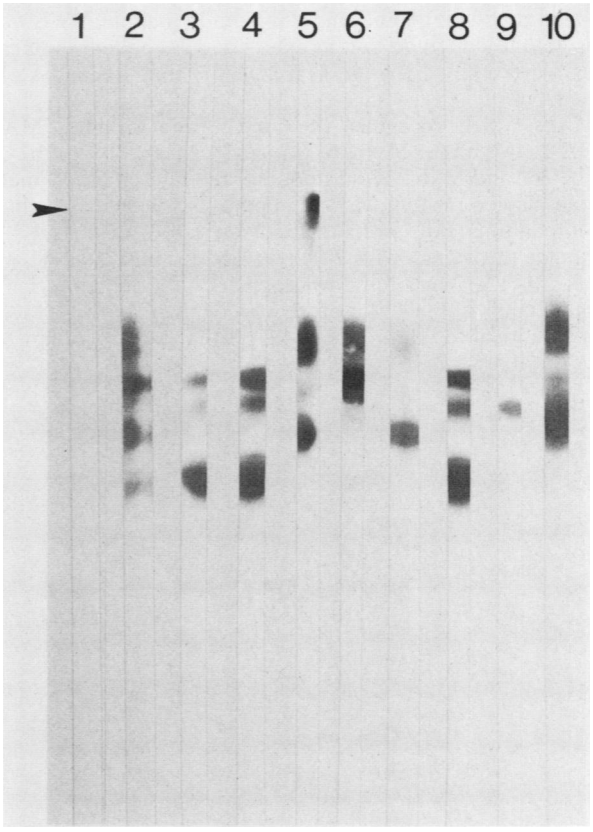

FIG. 1. Immunopeptide map of bp37$^{v\text{-}myb}$. Purified bp37$^{v\text{-}myb}$ was digested with *Staphylococcus aureus* V8 protease by the method of Cleveland et al. (3). One hundred nanograms of protease was used per 1-cm-wide well of the stacking gel. Digested bp37$^{v\text{-}myb}$ was fractionated on a 15% SDS-polyacrylamide gel, and the peptide fragments were electroblotted onto nitrocellulose paper. The nitrocellulose blot was cut into longitudinal strips, and each strip was probed with antibody as follows: normal rabbit serum (strip 1), rabbit anti-bp37$^{v\text{-}myb}$ serum (strip 2), Myb2-2 (strip 3), Myb2-3 (strip 4), Myb2-6 (strip 5), Myb2-7 (strip 6), Myb2-10 (strip 7), Myb2-32 (strip 8), Myb2-36 (strip 9), and Myb2-37 (strip 10). Arrow, Position at which undigested bp37$^{v\text{-}myb}$ would run. Undigested bp37$^{v\text{-}myb}$ appears only in strip 5, probably because of variability in digestion of bp37$^{v\text{-}myb}$ in different sample wells of the stacking gel.

TABLE 2. Blocking of Myb2 MAb binding by other Myb2 MAbs

^{3}H-MAb	Reaction by MAb[a]							
	Myb2-2	Myb2-3	Myb2-6	Myb2-7	Myb2-10	Myb2-32	Myb2-36	Myb2-37
Myb2-2	+	+	−	+	−	+	−	−
Myb2-3	+	+	−	−	ND	+	−	−
Myb2-6	−	−	+	−	ND	−	−	±
Myb2-7	−	−	−	+	ND	+	−	−
Myb2-10	−	−	−	ND	+	−	−	−
Myb2-32	+	+	−	−	ND	+	−	−
Myb2-36	−	−	−	−	−	±	+	−
Myb2-37	−	−	±	−	−	−	−	+

[a] Blocking reaction symbols are as follows: +, blocking; ±, partial (i.e. < 50%) blocking; −, no blocking; ND, not determined.

blocked each other, and Myb2-32 blocked Myb2-36 but not vice versa.

It thus appears that one part of the v-*myb* protein constituted a particularly strong immunogenic region, as it was recognized by three of the eight MAbs so far tested. Nonetheless, the determinants recognized by some other Myb2MAbs were spread far enough apart to be distinguished by blocking and immunopeptide mapping. We were thus able to conclude that at least six independent antigenic sites were defined by the Myb2 MAbs that we had so far tested.

MAbs which recognize the v-*myb* gene product. MAbs Myb2-2, Myb2-3, Myb2-6, Myb2-7, Myb2-10, Myb2-32, Myb2-36, and Myb2-37 all immunoprecipitated a [^{35}S]methionine-labeled protein with an apparent molecular weight of 45 kilodaltons (kd) specifically from BM-2 cells (Fig. 2), although the signal with MAbs Myb2-6 and Myb2-36 was weak. Myb2-37 in addition recognized a 37-kd protein. V8 peptide maps of the 45-kd protein recognized by these MAbs were identical to that of the 45-kd protein p45^{myb} recognized by established rabbit anti-*myb* serum (10) (see Fig. 4). Removal of p45^{myb} from cell lysates by immunoadsorption with rabbit antiserum specifically removed the p45 recognized by the MAbs but left behind the p37 recognized by Myb2-37 (data not shown). We therefore concluded that all eight of these MAbs recognized the v-*myb* gene product.

MAbs recognizing the c-*myb* gene product. The avian lymphoma line R2B has been shown to express elevated levels of c-*myb* mRNA (7). Figure 3 shows the results of immunoadsorption of a [^{35}S]methionine-labeled R2B cell lysate with the Myb2 MAbs. MAbs Myb2-2, Myb2-3, Myb2-7, and Myb2-32 clearly recognized a 75-kd protein similar to that seen by the rabbit anti-*myb* serum. Peptide mapping demonstrated that the molecules seen by these MAbs were indeed the c-*myb* gene product (Fig. 4). MAb Myb2-37 reproducibly recognized several components in R2B cells in addition to p37. However, V8 peptide mapping showed that, of these several components, the 75-kd protein was p75$^{c\text{-}myb}$. The identity of the other proteins recognized by Myb2-37 in R2B cells is unknown. No p75$^{c\text{-}myb}$ was

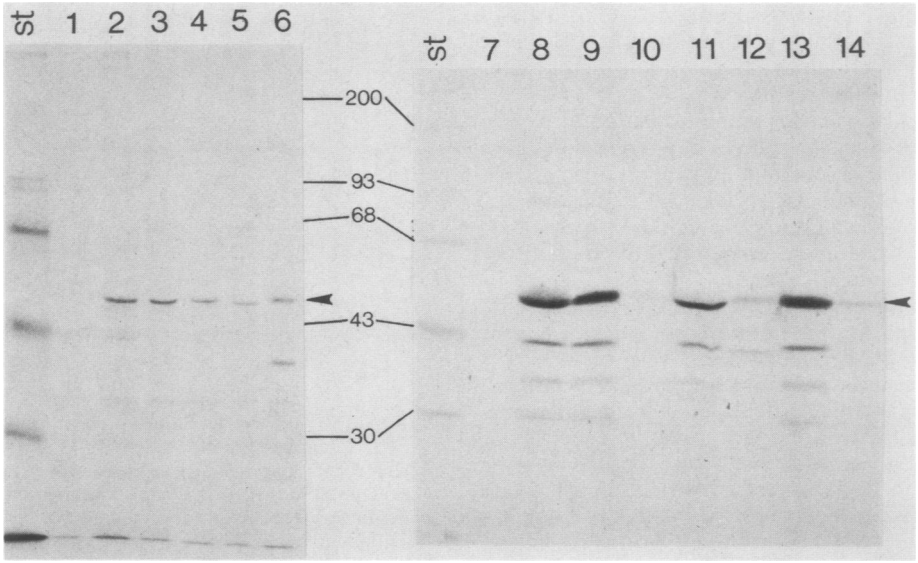

FIG. 2. Immunoprecipitation of p45$^{v\text{-}myb}$ from BM-2 cells. BM-2 myeloblasts were labeled with [^{35}S]methionine and immunoprecipitated with various antibodies as described in the text. The figure shows the results of two separate experiments with gels containing the following: normal rabbit serum (lane 1), Myb2-2 antigen (lane 2), Myb2-3 antigen (lane 3), Myb2-7 antigen (lane 4), Myb2-32 antigen (lane 5), Myb2-37 antigens (lane 6), normal mouse serum (lane 7), Myb2-2 antigen (lane 8), Myb2-3 antigen (lane 9), Myb2-6 antigen (lane 10), Myb2-7 antigen (lane 11), Myb2-10 antigen (lane 12), Myb2-32 antigen (lane 13), and Myb2-36 antigen (lane 14). st, Gels containing molecular weight markers for myosin (200 kd), phosphorylase (92.5 kd), bovine serum albumin (68 kd), ovalbumin (43 kd), and carbonic anhydrase (30 kd). Arrow, p45$^{v\text{-}myb}$. We suspect that some of the other bands prominent in lanes 8 to 14, particularly that migrating at ca. 40 kd, may be breakdown products of p45$^{v\text{-}myb}$.

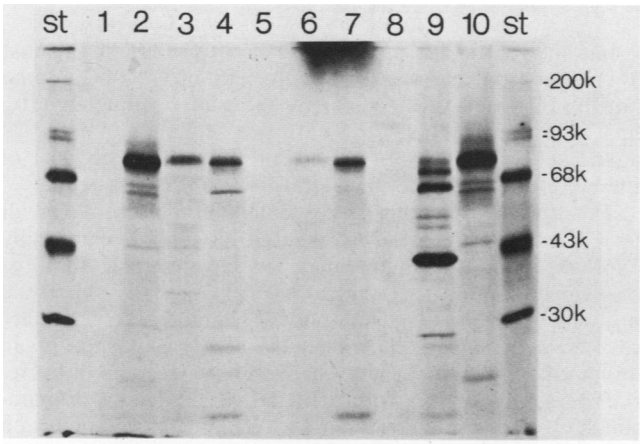

FIG. 3. Immunoprecipitation of c-*myb* gene products from R2B lymphoma cells. R2B avian leukosis virus-induced lymphoma cells were labeled with [^{35}S]methionine and immunoprecipitated as described in the text. Antibodies used are as follows: standards (st; see the legend to Fig. 2), normal rabbit serum (lane 1), rabbit anti bp37$^{v\text{-}myb}$ serum (lane 2), Myb2-2 (lane 3), Myb2-3 (lane 4), Myb2-6 (lane 5), Myb2-7 (lane 6), Myb2-32 (lane 7), Myb2-36 (lane 8), Myb2-37 (lane 9), rabbit anti-bp37$^{v\text{-}myb}$ (lane 10), and standards (as in lane 1).

observed to be precipitated by MAbs Myb2-6 or Myb2-36, even on overexposed autoradiographs (Fig. 3).

Of interest is the observation that the rabbit antiserum and MAbs Myb2-3 and Myb2-32 immunoprecipitated two components of similar size from R2B cells, whereas MAbs Myb2-2 and Myb2-7 recognized only the higher-molecular-weight component. This phenomenon was more clearly shown when the immunoprecipitated c-*myb* products were run on a gradient SDS-polyacrylamide gel (Fig. 5). At present we do not know the significance of this c-*myb* doublet. V8 peptide maps of both upper and lower bands were identical (data not shown), suggesting that differences between the two c-*myb* components are slight. This doublet was not observed in other cell lines expressing c-*myb* that we have tested. Instead, a single 75-kd component was observed, which was recognized by all of the MAbs described in this section as reactive to R2B p75$^{c\text{-}myb}$ (data not shown).

Immunoprecipitation of the E26 oncogene product. PJ212 is a chicken myeloblast cell line transformed by the E26 virus. The E26 virus causes erythroblastosis and myeloblastosis in chickens. The virus genome contains two regions which are thought to be responsible for oncogenicity, a region homologous to part of the AMV v-*myb* gene (*myb*^{a}) and an unrelated region called v-*ets* (13, 18). The transforming sequences code for a single 135-kd protein (p135$^{gag\text{-}myb\text{-}ets}$) which presumably carries out the transforming function of the virus.

Figure 6 shows that MAbs Myb2-2, Myb2-3, Myb2-7, and Myb2-32 recognized p135$^{gag\text{-}myb\text{-}ets}$ in [^{35}S]methionine-labeled PJ212 cell lysates, whereas Myb2-6 and Myb2-36 did not. Interestingly, Myb2-37 also failed to detect p135, suggesting that the MAb might be specific for an epitope not present in the *myb* domain of p135$^{gag\text{-}myb\text{-}ets}$.

Analysis of the 37-kd protein recognized by Myb2-37. We sought more information about the 37-kd protein which was recognized along with v-*myb* and c-*myb* gene products by Myb2-37. We determined that Myb2-37 was recognizing an epitope on both p45^{myb} and p37 and not simply immunoprecipitating a complex of p37 and p45$^{v\text{-}myb}$. This was shown by the fact that Myb2-37 recognizes bands at both 45 and 37 kd on Western blots of BM-2 cell lysates fractionated on an SDS-polyacrylamide gel. The other anti p45^{myb} MAbs recognized only a 45-kd protein in such assays (Fig. 7).

Both immunoblotting (Fig. 7) and immunoprecipitation (Fig. 2) of p37 from [^{35}S]methionine-labeled BM-2 cell extracts suggested that the level of p37 was roughly comparable with that of p45$^{v\text{-}myb}$. Immunofluorescence microscopy demonstrated that p37 was localized mainly in the nucleus of all tested cells (K.-H. Klempnauer, personal communication) and was released from isolated nuclei after treatment with DNase and high salt. In this regard, p37 behaved in the same way as did p45$^{v\text{-}myb}$, and it behaved differently from the v-*myc* and c-*myc* gene products and several other nuclear

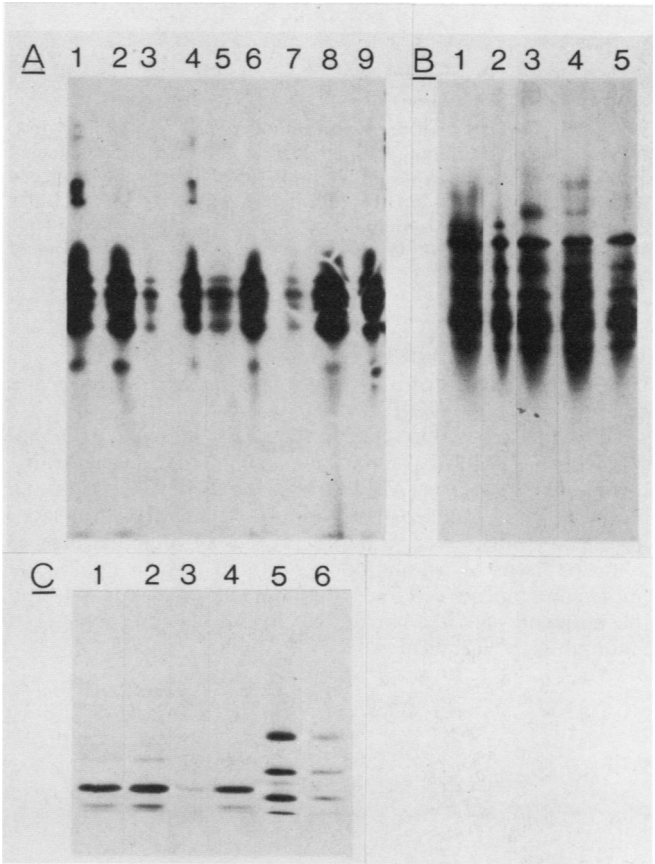

FIG. 4. V8 protease fingerprints of p45$^{v\text{-}myb}$, p75$^{c\text{-}myb}$, and p37 antigens recognized by Myb2 MAbs. Antigens were immunoprecipitated from [^{35}S]methionine-labeled BM-2 myeloblast (p45$^{v\text{-}myb}$) or R2B lymphoma cells (p75$^{c\text{-}myb}$) and digested with 10 ng of V8 protease (A and B) or 100 ng of V8 protease (C) per well by the method of Cleveland et al. (3). Digested fragments were fractionated on 15% (A and B) or 10 to 20% linear gradient (C) SDS-polyacrylamide gels. (A) Antigens precipitated from BM-2 myeloblasts were as follows: rabbit anti-bp37$^{v\text{-}myb}$ antigen (lane 1), Myb2-2 antigen (lane 2), Myb2-3 (lane 3), Myb2-6 (lane 4), Myb2-7 (lane 5), Myb2-10 (lane 6), Myb2-36 (lane 7), Myb2-32 (lane 8), and Myb2-37 (lane 9). (B) Antigens precipitated from R2B lymphoma cells were as follows: rabbit anti-bp37$^{v\text{-}myb}$ antigen (lane 1), Myb2-32 antigen (lane 2), Myb2-2 antigen (upper p75$^{c\text{-}myb}$ band; lane 3), Myb2-2 antigen (lower p75$^{c\text{-}myb}$ band; lane 4), and Myb2-37 75-kd antigen (lane 5). (C) Comparison of p45$^{v\text{-}myb}$ and p37 antigens was as follows: mouse anti-bp37$^{v\text{-}myb}$ antigen from BM-2 cells (lane 1), Myb2-2 antigen from BM-2 cells (lane 2), Myb2-6 antigen from BM-2 cells (lane 3), Myb2-37 antigen from BM-2 cells (lane 4), p37 antigen from BM-2 cells (lane 5), and p37 antigen from R2B cells (lane 6).

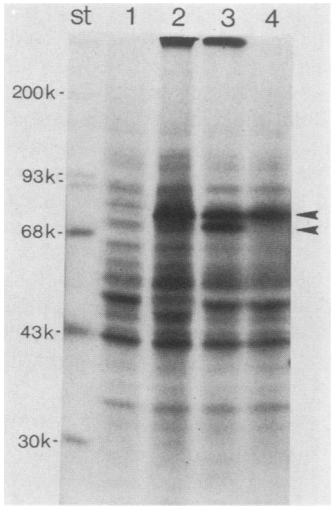

FIG. 5. Gradient SDS-polyacrylamide gel of c-*myb* antigens. Antigens were immunoprecipitated from [^{35}S]methionine-labeled R2B lymphoma cells as described in the legend to Fig. 4. Antigens were then fractionated on 10 to 20% linear gradient gel. Antibodies used were as follows: standards (st; as for Fig. 3), normal mouse serum (lane 1), mouse anti-bp37$^{v\text{-}myb}$ (lane 2), Myb2-32 (lane 3), and Myb2-2 (lane 4).

components (unpublished data). Mapping of p37 with V8 protease failed to reveal homology to p45$^{v\text{-}myb}$ (Fig. 4). However, V8 maps of p37 from a number of different avian cell lines were identical.

Upon further analysis, we found p37 in all avian cells that we tested, including avian erythroblastosis virus-transformed erythroblasts, MC29-transformed fibroblasts, avian leukosis virus-induced lymphomas, and normal chicken fibroblasts. Moreover, a 37-kd protein of identical size to avian p37 was also immunoprecipitated from a number of human and murine cell lines (data not shown). Thus, p37 and the antigenic site recognized by Myb2-37 were conserved among higher vertebrates.

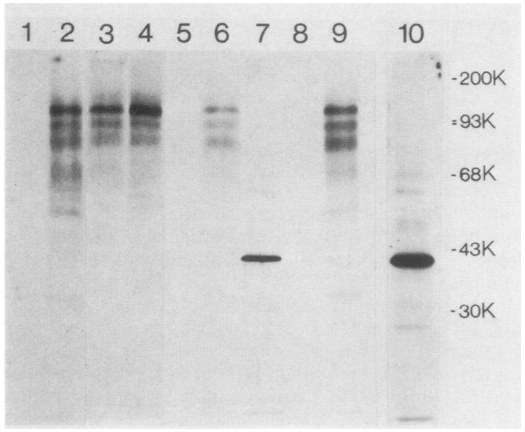

FIG. 6. Immunoprecipitation of antigens from E26-transformed myeloblasts. E26-transformed myeloblasts (PJ212) were labeled and lysed as described in the text. Samples of the lysate were then immunoprecipitated with the following antibodies: normal mouse serum (lane 1), rabbit anti-bp37$^{v\text{-}myb}$ (lane 2), Myb2-2 (lane 3), Myb2-3 (lane 4), Myb2-6 (lane 5), Myb2-7 (lane 6), Myb2-37 (lane 7), Myb2-36 (lane 8), Myb2-32 (lane 9), and overexposed autoradiogram of Myb2-37 from lane 7 (lane 10). The two accompanying bands below p135$^{gag\text{-}myb\text{-}ets}$ may be breakdown products. Their appearance varied from experiment to experiment.

DISCUSSION

Immunogenicity of bp37$^{v\text{-}myb}$. We have raised MAbs against a bacterially expressed fusion protein bp37$^{v\text{-}myb}$, a large portion of which is coded for by the v-*myb* sequence (10). bp37$^{v\text{-}myb}$ also contains an N-terminal sequence of some 22 amino acids from the N terminus of HGH; thus, ca. 96% of the bp37$^{v\text{-}myb}$ sequence is coded for by v-*myb* sequence.

The bp37$^{v\text{-}myb}$ protein is extremely immunogenic in all tested mouse strains. This is unusual among bacterially expressed fusion proteins that we have tested. In most cases, we found it necessary to purify bacterially expressed proteins by preparative SDS-polyacrylamide gel electrophoresis before immunization because of the existence of an immunodominant 33-kd protein present in extracts of bacterial lysates that are insoluble in NP-40 (Evan, in preparation). Most of the hybridoma supernatants that reacted on control (but not on test) ELISA plates in our screening of the Myb2 fusion were specific for this 33-kd protein (data not shown).

The range of antigenic sites recognized on bp37$^{v\text{-}myb}$ by our MAbs appears to be quite broad. Nonetheless, at least one region on the molecule seems to be exceptionally immunogenic as suggested by the observation that three of the eight tested MAbs block the binding of each other and share an identical immunopeptide map. In our experience, the paucity of good antigenic sites on bacterially expressed fusion proteins is more common that not.

Although the antigenic sites recognized by MAb Myb2-2, Myb2-3, and Myb2-32 are close enough to allow them to interfere with each other in binding, we have been able to partially discriminate between the binding sites of MAbs.

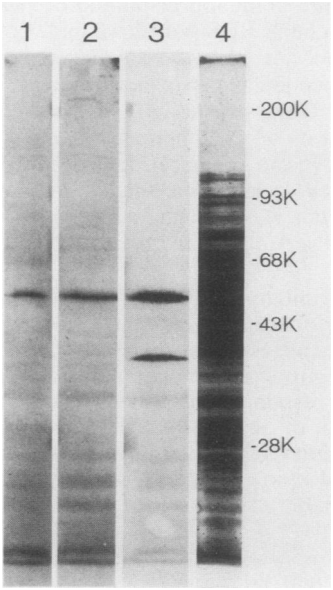

FIG. 7. Immunoblot of BM-2 myeloblast cell lysates fractionated on a 12.5% SDS-polyacrylamide gel. BM-2 cells were lysed in SDS sample buffer, sheared through a 23-gauge needle, and boiled for 5 min. Cell equivalents (5×10^6) of the lysate were loaded on each gel. The SDS gel was electroblotted onto nitrocellulose paper and probed with antibodies as described in the text. Molecular weights were determined with Bethesda Research Laboratories prestained molecular weight markers which were fractionated in lanes alongside those containing cell lysates. Antibodies used were as follows: rabbit anti-bp37$^{v\text{-}myb}$ serum (lane 1), Myb2-2 (lane 2), Myb2-37 (lane 3), and total protein stained with India ink (lane 4).

TABLE 3. Specificities of Myb2 MAbs

Antibody	Reaction against protein				
	bp37$^{v\text{-}myb}$	p45$^{v\text{-}myb}$	p75$^{c\text{-}myb}$	p135$^{gag\text{-}myb\text{-}ets}$	p37
Myb2-2	+	+	+	+	−
Myb2-3	+	+	+(d)[a]	+	−
Myb2-6	+	+	−	−	−
Myb2-7	+	+	+	+	−
Myb2-10	+	+	ND	ND	−
Myb2-32	+	+	+(d)	+	−
Myb2-36	+	+	−	−	−
Myb2-37	+	+	+	−	+
Mouse anti-bp37$^{v\text{-}myb}$ [b]	+	+	+	+	−
Rabbit anti-bp37$^{v\text{-}myb}$ [b]	+	+	+(d)	+	−

[a] (d), Recognizes two p75$^{c\text{-}myb}$ components in R2B lymphoma cells.
[b] Polyclonal antiserum.

For example, whereas Myb2-2 immunoprecipitates only the upper of the two c-*myb* bands from R2B cells, Myb2-3 as well as Myb2-32 recognize both bands. Also, Myb2-2 binding to bp37$^{v\text{-}myb}$ is partially but reproducibly blocked by Myb2-7, unlike the binding of Myb2-3 and Myb2-32. Thus, of the seven MAbs (Myb2-2, Myb2-3, Myb2-6, Myb2-7, Myb2-32, Myb2-36 and Myb2-37), only two (Myb2-3 and Myb2-32) have indistinguishable antigenic specificities.

Immunoprecipitation of *myb* gene products. All of the tested Myb2 MAbs immunoprecipitate p45$^{v\text{-}myb}$ from BM-2 cells. All but two of these appear also to recognize the p75$^{c\text{-}myb}$ gene product. It is possible that two MAbs, Myb2-6 and Myb2-36, that do not immunoprecipitate p75$^{c\text{-}myb}$ recognize determinants coded for by those DNA sequences which are specific to the v-*myb* and not the c-*myb* gene (9). We have observed, however, that Myb2-6 and Myb2-36 immunoprecipitate much less p45$^{v\text{-}myb}$ from equivalent samples of BM-2 cell lysate than do the other Myb2 MAbs, which may be due to the low affinity of the former for the antigen. If MAbs Myb2-6 and Myb2-36 also immunoprecipitate proportionately lower amounts of p75$^{c\text{-}myb}$, it is possible (although we feel it unlikely) that we would not detect the weak p75$^{c\text{-}myb}$ signal, even on heavily overexposed autoradiograms.

MAbs Myb2-2, Myb2-3, Myb2-7, and Myb2-32 recognize the p135$^{gag\text{-}myb\text{-}ets}$ gene product coded by the E26 virus oncogenic sequence. E26 v-*myb* sequence shares only 800 of the 1,035 nucleotides of the AMV v-*myb* coding sequence (18); thus, we are able to assign the binding sites of these MAbs to the region coded for by the 800-nucleotide homology. MAb Myb2-37 recognizes both AMV v-*myb* and c-*myb* products but not the E26 v-*myb* gene product. We thus conclude that Myb2-37 probably recognizes an epitope coded for within the 235 3′ nucleotides (coding for ca. 78 amino acids) of AMV v-*myb* which are absent in E26 v-*myb*. MAbs Myb2-6 and Myb2-36 do not immunoprecipitate the p135$^{gag\text{-}myb\text{-}ets}$ protein. Because the E26 v-*myb* sequence more closely resembles that of c-*myb* than AMV v-*myb* does, this further suggests that MAbs Myb2-6 and Myb2-36 recognize sequences specific for AMV v-*myb*. A summary of the specificities of the Myb2 MAbs is shown in Table 3.

Studies with these MAbs have some bearing on the current controversy over the size of the c-*myb* gene product. Boyle et al. (1), using polyclonal antisera raised against synthetic peptides whose sequences were predicted from those of AMV v-*myb*, have identified a 110-kd protein in cells expressing c-*myb*. In contrast, Klempnauer et al. (10), using antisera raised against the same bacterially expressed bp37$^{v\text{-}myb}$ as described above have identified the c-*myb* gene product as a 75-kd protein. Isolation in this experiment of five independent MAbs which recognize between them at least three independent epitopes binding both p45$^{v\text{-}myb}$ and p75 suggests that the homology between the two proteins is extensive. Thus, p75 is very likely the product of the c-*myb* gene. In none of our studies have we observed any recognition of a 110-kd protein by our MAbs.

The 37-kd protein recognized by MAb Myb2-37. MAb Myb2-37 recognizes p45$^{v\text{-}myb}$, p75$^{c\text{-}myb}$, and the 37-kd protein p37. The specificity of Myb2-37 for both p45$^{v\text{-}myb}$ and p37 is demonstrated by the recognition of both proteins by the MAb on immunoblots of BM-2 cell lysates fractionated on an SDS-polyacrylamide gel. However, V8 protease fingerprinting shows that p37 has no obvious homology with any *myb* gene products.

Immunoprecipitations of subcellular fractions from [^{35}S]methionine-labeled cells suggest that p37 is located mainly in the nucleus. The p37 in the nucleus, like the gene products of AMV v-*myb* and c-*myb*, is released when isolated nuclei are treated with DNase and high salt (G. Ramsay, personal communication; unpublished data).

Both p37 and the Myb2-37 binding site on p37 are phylogenetically highly conserved among avians and mammals. This might perhaps argue for the importance of the Myb2-37 epitope, or its immediate environs, in the function of p37. Although it is tempting to speculate on the fact that a highly conserved site on a highly conserved nuclear protein is immunologically related to a site on v-*myb* and c-*myb* gene products, the number of irrelevant cross-reactions which have been observed with various MAbs makes us wary of attempting to interpret this phenomenon.

We have not yet attempted to identify p37 with known chromatin components. We do, however, know that p37 is a fairly abundant protein present at about the same level as p45$^{v\text{-}myb}$ in BM-2 cells, and its mobility on SDS-polyacrylamide gels argues against its being a histone.

General properties of monoclonal antibodies to bacterially expressed antigens. Using a similar approach to our own, Lipsich et al. (14) and Parsons et al. (19) report having successfully used bacterially expressed pp60^{src} to produce MAbs specific for the native product of the *src* gene. An increasingly popular and alternative approach to the use of bacterially expressed immunogens has been developed by Lerner and colleagues (12). In this method, synthetic peptides, whose sequences are predicted from nucleic acid sequence data, are used as immunogens. Peptide-specific MAbs have proven very effective in recognizing native oncogene products (17, 20).

Both of the above approaches are alternatives to immunizing with native proteins which are often difficult to purify in enough quantity for use as immunogens. It is noteworthy, however, that a number of major antigenic determinants present on native proteins may not be found on bacterially expressed proteins or synthetic peptides, particularly those reflecting high degrees of tertiary structure. Undoubtedly, the reverse case is also true, and some determinants on bacterially expressed proteins or synthetic peptides may be unavailable for binding on native proteins.

The greater variety of epitopes present on bacterially expressed proteins appears to be their most obvious advantage over synthetic peptides as immunogens. In our experience, however, we have found many bacterially expressed proteins to be poorly immunogenic and, moreover, to carry immunodominant determinants which are sometimes not

found on their native protein counterparts. This may reflect the fact that large polypeptides may assume conformations in solution which are not related to those found in their native protein counterparts. Not surprisingly in such cases, few, if any, MAbs raised against the bacterial antigen will recognize the native antigen. In contrast to these observations, MAbs raised against short synthetic peptides, coupled to immunogenic carrier proteins, cross-react with native proteins with surprising frequency (17). We are currently investigating ways of making bacterially expressed fusion proteins more immunogenic.

ACKNOWLEDGMENTS

We thank Karl-Heinz Klempnauer, Marty Privalsky, Gary Ramsay, Craig Morita, Ling-Chun Chen, Geoff Symonds, Randy Schatzman, and Carol Nottenberg for indispensable advice and discussion; Art Levinson, John McGrath, and Wendy Colby (Genentech, Inc.) for bacterially expressed bp37$^{v\text{-}myb}$ extracts, and Janine Marinos for preparing the manuscript.

This work was supported by grants from the National Cancer Institute and the American Cancer Society. G.I.E. was supported by fellowships from the Medical Research and the Science and Engineering Research Councils of Great Britain.

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