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Monoclonal Antibodies to Feline Sarcoma Virus *gag* and *fes* Gene Translational Products

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A series of hybridomas have been isolated which produce monoclonal antibodies directed against polyprotein gene products of the Gardner, Snyder-Theilen, and McDonough strains of FeSV. Within these are representatives of several immunoglobulin classes including IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgM. Antibody produced by one hybridoma recognizes immunologic determinants localized within an FeLV *gag* gene structural component (p15) common to polyproteins encoded by all three FeSV isolates whereas antibody produced by a second is specific for p30 determinants unique to $P170^{gag\text{-}fms}$. Additional hybridomas secrete antibody directed against v-*fes*-encoded determinants common to the Gardner and Snyder-Theilen FeSV-encoded polyproteins. GA $P110^{gag\text{-}fes}$ and ST $P85^{gag\text{-}fes}$ immunoprecipitated by antibody directed against p15 exhibit tyrosine-specific protein kinase activity but lack such activity when precipitated by antibody specific for their acquired sequence (v-*fes*) components.

Key words: feline sarcoma virus proteins, v-*fes*, v-*fms*, tyrosine, monoclonal antibodies, tyrosine phosphorylation

Several independent isolates of feline sarcoma virus (FeSV) have been described [1–3]. Each, in the presence of an appropriate type C helper virus, transforms embryo fibroblasts in cell culture and induces fibrosarcomas in vivo. The transforming activities of these viruses are associated with acquired genetic sequences of cellular origin. These include the v-*fes* gene common to the Gardner (GA) and Snyder-Theilen (ST) strains of feline sarcoma virus (FeSV) [4–8], and the v-*fms* gene which is unique to McDonough (McD) FeSV [4,9,10]. The translational products of these acquired cellular sequences are expressed covalently linked to amino terminal FeLV structural components in the form of high-molecular-weight (M_r) polyproteins [11,12]. These include an 85,000 M_r polyprotein encoded by the prototype isolate of ST FeSV [11,12,8], and 110,000 and 170,000 M_r polyprotein gene products of GA FeSV [4,8] and McD FeSV [4,10], respectively. The ST and GA FeSV-encoded polyproteins contain FeLV p15 and p12 amino terminal structural components [4,8,11,12], whereas the McD FeSV translational product, designated $P170^{gag\text{-}fms}$ according to convention [13], contains p15, p12, and p30 [4,10].

Functional analysis of the GA and ST FeSV gene products has led to the identification of a tyrosine-specific protein kinase activity [14–16]. The involvement

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analysis of a series of transformation-defective (td) viral mutants [17,18], and single preferred tyrosine phosphorylation acceptor sites have been described [19,20]. Analogous enzymatic activities have been described in association with several other well-defined transforming proteins [21] including those encoded by an avian retrovirus transformation-specific gene (v-*fps*) genetically related to the mammalian v-*fes* gene [22]. In contrast, the *fms* gene product, represented within the McD FeSV-encoded polyprotein $P170^{gag\text{-}fms}$, lacks detectable enzymatic activity when assayed under similar conditions [18]. In an effort to better define the *fes* and *fms* gene products, the present study was undertaken to develop hybridomas producing antibody with specificity for both the viral and acquired sequence-encoded components of the available FeSV-encoded polyproteins.

MATERIALS AND METHODS

Cells and Viruses

Cell lines used have been described previously and include a highly contact-inhibited Fisher rat embryo line, FRE 3A [23], as well as subclones of FRE 3A cells nonproductively transformed by the GA, ST, and McD strains of FeSV, designated GA-FeSV FRE C14, ST-FeSV FRE C110, and SM-FeSV FRE C13, respectively [18,23]. In addition, CCL64 mink cells and nonproductively transformed subclones designated GA-FeSV 64 C12, ST-FeSV 64 C156, and SM-FeSV 64 C115 [18,23] were used. A clonal isolate of FeLV subgroup A, propagated on CCL64 mink cells, has been previously described [8]. The Lou rat myeloma line, Y3-Ag 1.2.3 [24] was a gift of C. Milstein, Cambridge, England.

Isolation of Hybridoma Clones

Weanling Fisher rats were immunized subcutaneously with 1.0×10^7 disrupted cells resuspended in 0.2 ml Freund's complete adjuvant and 2 weeks later challenged with 1.0×10^6 live cells. Animals in which large tumors developed at the site of inoculation and subsequently regressed were reimmunized with 1.0×10^7 live cells every 2 weeks thereafter for a period of 3–4 months. Three to 5 days following the final immunization, spleens were removed and splenic lymphocytes (2.0×10^8) were fused with an equal number of Y3-Ag 1.2.3 rat myeloma cells and maintained in culture according to previously described procedures [24]. Following 10–12 days' incubation at 37°C, culture fluids were harvested, pooled in groups of 3–10, and screened by immunoprecipitation and SDS-PAGE analysis. Cultures scored as positives were subcloned initially at a concentration of five cells/well and subsequently as single cell clones.

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE

Tissue culture cells were radioactively labeled by incubation for 2 h in medium containing either ^{32}P-orthophosphate or ^{35}S-methionine, cell extracts were prepared, and immunoprecipitations were performed according to previously described methods [10].

In Vitro Phosphotransferase Reactions

Tissue culture cells (1.0×10^7) were disrupted by repeated aspiration through a 25-gauge needle in 5 ml of 10 mM Tris-HCl, pH 7.2, 200 mM NaCl, 1.0% Triton

X-100, and 5 mM $MnCl_2$ (TBSTM), and lysates were clarified by centrifugation at 100,000g for 1 hr. Following addition of 10-μl specific antiserum, lysates were incubated for 18 hr at 4°C, 100 μl of a 10% suspension of protein-A-Sepharose was added, and incubation continued for 2 hr at 4°C. Immunocomplexes were collected by centrifugation for 10 min at 2,000g, washed in TBSTM buffer, resuspended in the same buffer containing 2.5 μCi [γ^{32}P]-ATP (2,000 Ci/mmol, New England Nuclear, Boston), and incubated for 10 min at 30°C. Reactions were terminated by addition of 3 ml ice-cold PSBTDS, and immunoprecipitates were washed and analyzed by SDS-PAGE as described above.

Immunoprecipitation of ^{125}I-Labeled FeLV Structural Proteins

FeLV structural proteins were purified, ^{125}I-labeled to high specific activity, and immunoprecipitates prepared according to previously described methods [25].

Immunodiffusion

Immunoglobulin subclasses were determined by the Ouchterlony technique using immunodiffusion agarose gels (Hyland Diagnostics, Deerfield, Illinois). Supernatant fluids from growing hybrid clones were dialyzed in phosphate-buffered saline (PBS) for 24 hr, lyophilized, and reconstituted in distilled water as tenfold concentrates. Typing antisera to the rat IgG subclasses and rat IgM were obtained from Miles Laboratories, Inc (Elkhart, Indiana). Precipitin lines were read after incubation for 24 hr at 37°C.

RESULTS

Spleen cells from 33 rats hyperimmunized with syngeneic nonproductively transformed cells were fused with the Y3-Ag 1.2.3 rat myeloma line, and cellular fluids from approximately 100–150 hybrid clones from each fusion were assayed for antibody against the appropriate viral polyprotein. Six clones including single hybridomas from spleen cells of rats immunized with either McD or GA FeSV-transformed cells and three from spleen cells of animals immunized with cells transformed by ST FeSV were scored as positives by immunoprecipitation SDS-PAGE analysis. An additional hybridoma, F118, secreted antibody specific for an 85,000 M_r cellular phosphoprotein. Culture fluids from each of the hybridomas, with the exception of F118, immunoprecipitated ^{35}S-methionine-labeled proteins of the expected molecular weight from both mink and rat FeSV-transformed, but not from nontransformed control cell lines. Representative immunoprecipitation data obtained using these reagents is presented in Figure 1.

As shown in Figure 2, each of the hybrid clones produced a single subclass of immunoglobulin: F72 and F40 producing IgG1, F113, IgG2b; and F123, IgG2a. Antibody produced by hybrid clone F118 was of IgG2c subclass. Although F115 immunoglobulin was significantly reactive with the broad IgG typing antiserum, it did not react with any of the more specific IgG subclass typing sera. Upon further analysis F115 immunoglobulin proved to be highly reactive with IgM typing antiserum, whereas immunoglobulins produced by each of the other hybrids were nonreactive (Fig. 3).

To define the specificities of the above described hybridomas, ascites fluids from each of the hybrid clones were analyzed for immunoprecipitation of ^{125}I-labeled

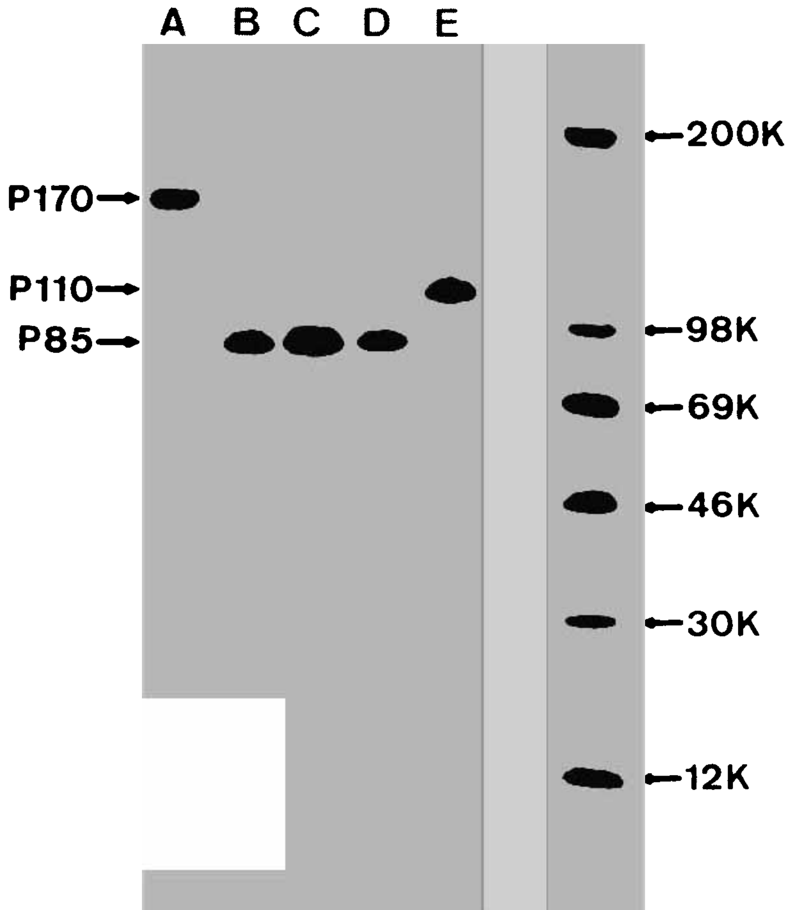

Fig. 1. Immunoprecipitation SDS-PAGE analysis of monoclonal antibodies in culture fluids of hybrid clones prepared from spleen cells of rats immunized with syngeneic FeSV-transformed cells. Cell lines including SM-FeSV FRE C13 (A), ST-FeSV FRE C110 (B–D), and GA-FeSV FRE C14 (E) were metabolically labeled by a 30-min incubation in ^{35}S-methionine-containing medium (100 μCi/ml), immunoprecipitated using 0.1 ml undiluted culture fluid from individual hybrids including F40 (A), F72 (B), F113 (C), F115 (D), and F123 (E) and analyzed by SDS-PAGE as described in Methods. Molecular weight standards include ^{14}C-labeled myosin (200,000), phosphorylase B (98,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome C (12,000).

FeLV *gag* and *env* gene-encoded structural proteins. As summarized in Table I, antibody produced by hybridoma F40 immunoprecipitated FeLV p30, whereas a second hybridoma clone, F72, produced antibody directed against p15. Of the three remaining hybridoma ascites fluids analyzed, none contained antibody that precipitated any of the ^{125}I-labeled FeLV structural proteins to a significant extent. To further define monoclonal antibodies produced by hybridoma clones F72 and F40, both were tested for immunoprecipitation of the polyprotein gene products of all three strains of FeSV. As summarized in Table II, ascites fluids from both clones precipitated P170$^{gag\text{-}fms}$, whereas only F72 antibody reacted with GA P110$^{gag\text{-}fes}$ and ST P85$^{gag\text{-}fes}$. This

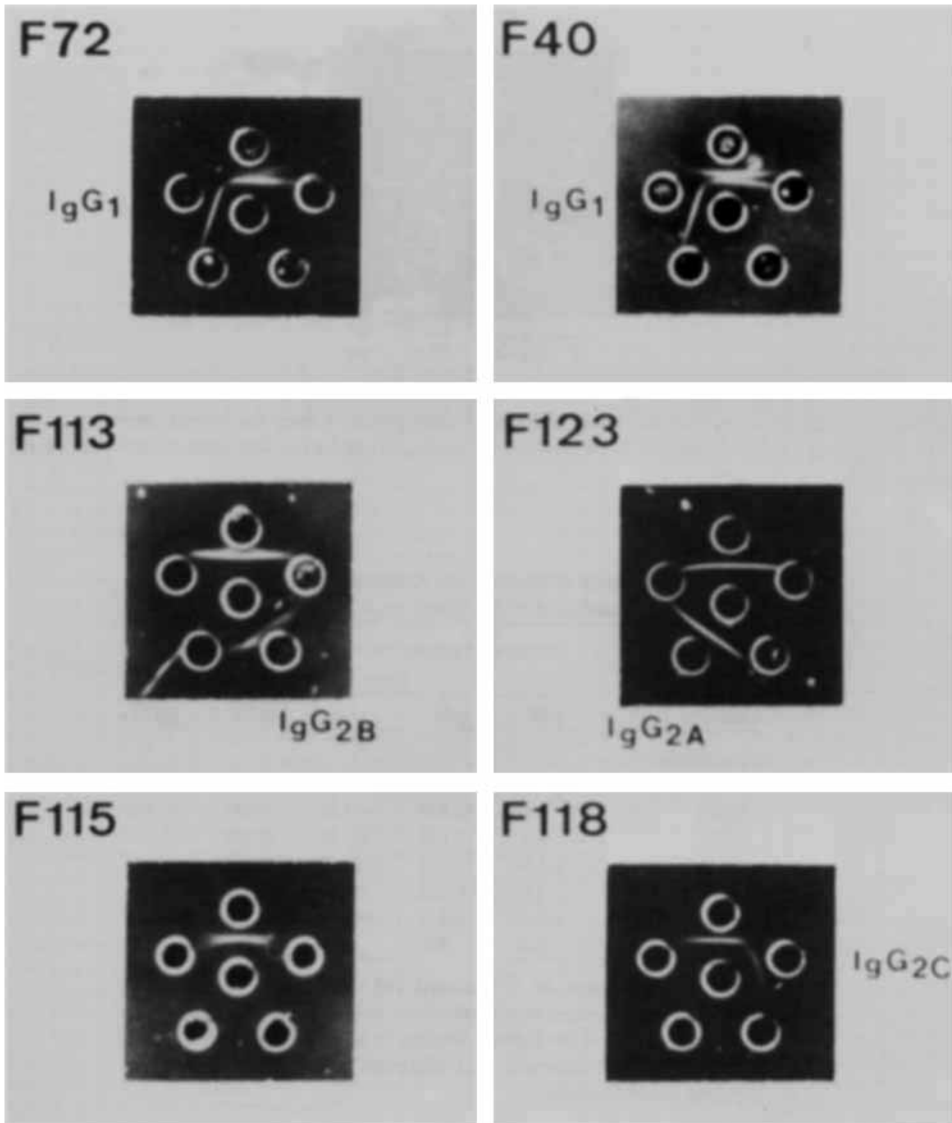

Fig. 2. Immunodiffusion patterns resulting from reaction of anti-rat IgG subclass typing antisera with monoclonal antibodies produced by hybrid cell lines. Starting counterclockwise from the top well, each of the six plates contain the following typing antisera: anti-IgG, anti-IgG_1, anti-IgG_{2a}, anti-IgG_{2b}, and anti-IgG_{2c}. The center wells of each plate contain the monoclonal antibody produced by hybridomas A) F72, B) F40, C) F113, D) F123, E) F115, and F) F118.

observation is consistent with the fact that, of these polyproteins, only FeSV $P170^{gag\text{-}fms}$ contains an extensive p30 component [10], and localizes the immunologic determinants recognized by F40 within the domain of p30 unique to $P170^{gag\text{-}fms}$. These findings identify FeLV structural components recognized by antibodies produced by two of the hybridoma clones, F40 and F72, as p15 and p30, respectively, and raised the possibility that the antibody produced by each of three remaining clones may be directed against v-*fes*-specific nonstructural components.

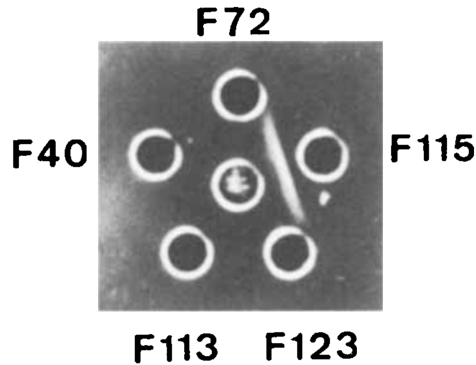

Fig. 3. Immunodiffusion patterns resulting from reaction of anti-rat IgM typing serum with monoclonal antibodies produced by hybrid cell lines. Immunoglobulin products from the hybrid clones F72, F40, F113, F123, and F115 are arranged counterclockwise starting from the top well. The center well contains anti-rat IgM.

TABLE I. Analysis of Monoclonal Antibodies for Immunoprecipitation of FeLV Structural Proteins

	Immunoprecipitation of ^{125}I-labeled proteins (titer $\log_{10}$)[a]				
Antibody	p15	p12	p30	p10	gp70
Hybridoma					
F40	<1.0	<1.0	6.2	<1.0	<1.0
F123	<1.0	<1.0	<1.0	<1.0	<1.0
F72	5.3	<1.0	<1.0	<1.0	<1.0
F113	<1.0	<1.0	<1.0	<1.0	<1.0
F115	<1.0	<1.0	<1.0	<1.0	<1.0
F118	<1.0	<1.0	<1.0	<1.0	<1.0
Goat anti-FeLV	3.8	3.2	5.3	2.1	3.9

[a]Immunoprecipitation of ^{125}I-labeled FeLV structural proteins was performed as described previously [25]. Antibody titers are expressed as the reciprocal of the highest dilution of ascites fluid at which 10% precipitation was observed, and represent mean values from three separate determinations.

To establish the apparent specificity of antibodies produced by hybridomas F113, F115, and F123 for GA and ST FeSV acquired sequence (v-*fes*) gene products, ascites fluids corresponding to each were tested for immunoprecipitation of GA $P110^{gag\text{-}fes}$, ST $P85^{gag\text{-}fes}$, $P170^{gag\text{-}fms}$, and FeLV $Pr65^{gag}$. As shown in Table II, hybridoma F72 ascites fluid precipitated all three ^{35}S-methionine-labeled viral polyproteins. In contrast, although F113, F115, and F123 specific monoclonal antibodies each precipitated GA $P110^{gag\text{-}fes}$, none of these recognized either $P170^{gag\text{-}fms}$ or FeLV $Pr65^{gag}$ (Table II). Moreover, monoclonal antibodies produced by all three hybridomas efficiently immunoprecipitated ST $P85^{gag\text{-}fes}$ (Table II). These findings argue that

TABLE II. Immunoprecipitation by Monoclonal Antibodies of Polyproteins Encoded by Independent FeSV Isolates

Hybridoma antibody	Immunoprecipitation of ^{35}S-methionine-labeled polyproteins and analysis by SDS-PAGE[a]			
	$Pr65^{gag}$	$P110^{gag\text{-}fes}$	$P85^{gag\text{-}fes}$	$P170^{gag\text{-}fms}$
F40	+	−	−	+
F123	−	+	+	−
F72	+	+	+	+
F113	−	+	+	−
F115	−	+	+	−
F118	−	−	−	−

[a]Immunoprecipitation analysis was performed by addition of 1.0 μl of ascites fluid, 0.1 ml goat anti-rat IgG, and 0.1 ml of a 10% suspension of protein A Sepharose to labeled extract and incubation for 18 h at 4°C. Immunoprecipitates were collected and analyzed by SDS-PAGE as previously described [23].

the immunological determinants recognized by F113, F115, and F123 map within acquired sequence-encoded components common to the GA and ST FeSV polyproteins.

It was of interest to examine the influence of antibody specifically directed against FeSV polyprotein v-*fes*-encoded component on the polyprotein-associated protein kinase activities. As shown in Figure 4, lanes A and E, respectively, ST $P85^{gag\text{-}fes}$ and GA $P110^{gag\text{-}fes}$ immunoprecipitated by F72 antibody exhibited readily detectable levels of in vitro autophosphorylation. In contrast, under similar conditions, no detectable labeling of $P170^{gag\text{-}fms}$ was observed (data not shown). When ST $P85^{gag\text{-}fes}$ was similarly tested following precipitation with ascites fluids from each of the three hybridomas directed against ST $P85^{gag\text{-}fes}$ nonstructural components, no detectable protein kinase was observed (Fig. 4, B–D). Analogous results were obtained upon analysis of GA $FeSV^{gag\text{-}fes}$ (Fig. 4, E–G), with the exception that following precipitation with one of the acquired sequence-specific monoclonal reagents, F113, a low but detectable level of in vitro phosphorylation was observed (Fig. 4, G). Further evidence for inhibition of the GA and ST FeSV-encoded protein kinases by monoclonal antibodies specifically directed against the polyprotein nonstructural components was obtained by immunoprecipitation of either GA $P110^{gag\text{-}fes}$ or ST $P85^{gag\text{-}fes}$ using 1:1 mixtures of F72 monoclonal antibody in combination with antibodies produced by the F113, F115, and F123 hybridomas. Protein kinase activity, as measured by autophosphorylation, was in each case markedly lower than that observed using F72 antibody alone.

DISCUSSION

In the present study we describe the isolation of a series of hybridomas producing high-titered antibody specific for polyprotein gene products of the GA, ST, and McD strains of FeSV. These include two hybridomas producing IgG_1 antibody against FeLV *gag* gene structural components, one of which is directed against FeLV p15, and the second, specific for FeLV p30. The FeLV p15 determinants recognized by

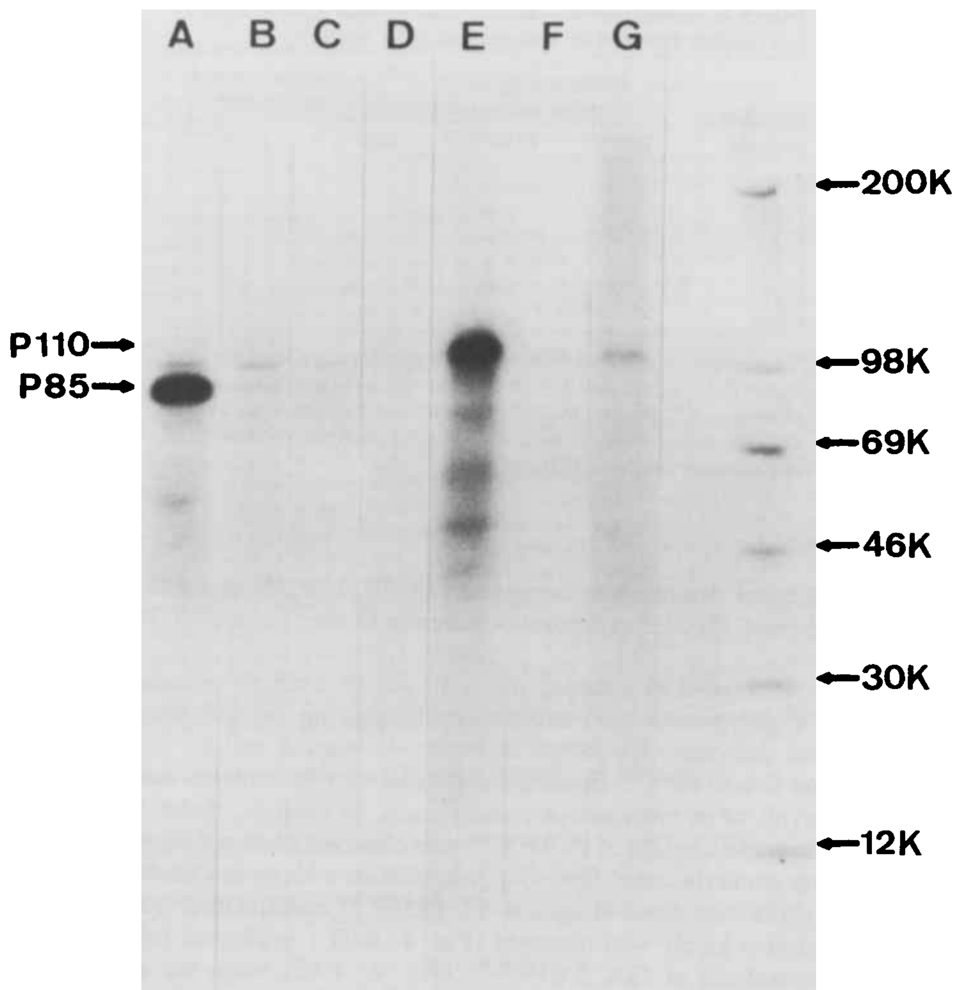

Fig. 4. Inhibition of the GA and ST FeSV-associated protein kinase activities following immunoprecipitation of polyproteins by monoclonal antibodies against their nonstructural components. Viral polyproteins were immunoprecipitated from extracts of ST-FeSV FRE C110 (A–D) and GA-FeSV 64 C12 (E–G) transformed cells, by 10 μl of a 1:1,000 dilution of ascites fluids from hybridomas F72 (A, E), F115 (B, F), F123 (C), and F113 (D, G), assayed for phosphotransferase activity using [γ^{32}P]-ATP as substrate, and analyzed by SDS-PAGE according to previously published procedures [15]. Molecular weight standards are as described in the legend to Figure 1.

the first of these clones are shared by polyprotein gene products of all three strains of FeSV whereas the p30 determinants recognized by the second are unique to $P170^{gag-fms}$. The three remaining hybridomas, one initially isolated from spleen cells of GA FeSV immunized rats and two from spleen cells of rats immunized with ST FeSV, lack detectable reactivity with FeLV structural proteins. Of these latter monoclonal antibody reagents, one is an IgG2a, one an IgG2b, and the remaining one an IgM. The specificities of these hybridomas are summarized in Figure 5.

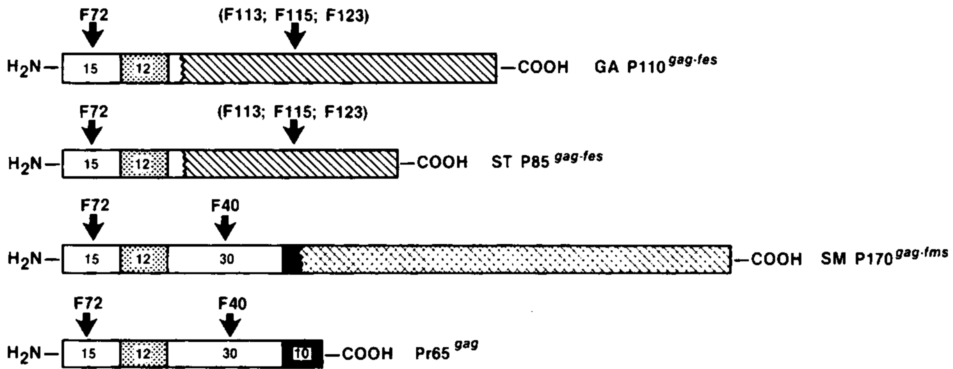

Fig. 5. Localization, within FeSV-encoded polyproteins, of antigenic determinants recognized by monoclonal antibodies with specificity for their structural (*gag*) and acquired sequence-encoded non-structural (v-*fes*) components.

Several lines of evidence indicate that monoclonal antibodies produced by hybridomas F113, F115, and F123 are specific for the acquired sequence (v-*fes*) components of the GA- and ST-encoded polyproteins. None of these three reagents exhibit detectable cross reactivity with FeLV Pr65^{gag}, or with any of the FeLV *gag* and *env* gene-encoded structural proteins. In contrast to polyproteins immunoprecipitated by F72 antibody, the F113-, F115-, and F123-precipitated polyproteins lack detectable enzymatic activity, and in appropriate mixing experiments direct inhibition of the protein kinase by these latter monoclonal reagents can be demonstrated. These findings are consistent with previous reports indicating hyperimmune sera directed against Rous sarcoma virus pp60^{src} to block autophosphorylation [26,27]. In contrast, in each case where antisera used for precipitation are directed against the structural components of retrovirus-encoded polyproteins, polyprotein phosphorylation is readily observed [21,28]. Immunoprecipitation of ST P85$^{gag\text{-}fes}$ by antibody secreted by F123, the one hybridoma originally isolated from spleen cells of a rat immunized with GA FeSV-transformed cells, argues that this reagent is specific for immunologic determinants mapping within the region of GA P110$^{gag\text{-}fes}$ shared with ST 85$^{gag\text{-}fes}$.

The demonstration of GA and ST FeSV polyprotein-associated protein kinase activities following immunoprecipitation by *gag* gene hybridoma antibody and specific inhibition of this activity by v-*fes*-specific antibody argues that the observed reactivities represented are intrinsic to the polyproteins. In contrast P170$^{gag\text{-}fms}$, when assayed following immunoprecipitation by antibody to its p15 and p30 structural components, lacked detectable protein kinase activity. In other studies, P170$^{gag\text{-}fms}$ was phosphorylated in tyrosine to a minor extent when coimmunoprecipitated with either the GA or ST FeSV-encoded polyproteins [29]. These findings are consistent with our previous results indicating that P170$^{gag\text{-}fms}$ lacks intrinsic protein kinase enzymatic activity [18] and may account for a reported low level of P170$^{gag\text{-}fms}$ phosphorylation in immunoprecipitates obtained with conventional hyperimmune antisera [30]. The immunologic reagents developed in the present study should be of value for further studies of the functional and structural organization of the various

FeSV polyprotein gene products. In particular, those monoclonal antibodies with specificity for the v-*fes* gene product may allow a determination of the role of this gene in naturally occurring tumors.

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