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A general method for the induction and screening of antisera for cDNA-encoded polypeptides: antibodies specific for a coronavirus putative polymerase-encoding gene

(Recombinant DNA; open reading frame vector; nonstructural viral proteins; cell-free protein synthesis; mouse hepatitis virus)

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SUMMARY

A prokaryotic vector, pGE374, containing the *recA* and *lacZ* genes, out-of-frame, was used for the expression of cDNA derived from the putative polymerase-encoding gene of the coronavirus mouse hepatitis virus strain A59 (MHV-A59). The pGE374/viral recombinant vector generates a tripartite bacterial/viral protein composed of a segment of the RecA protein at the N terminus, the coronaviral sequences in the middle, and an enzymatically active β-galactosidase at the C terminus. Rabbits immunized with such recombinant proteins generated antibodies to the MHV-A59 portion of the tripartite protein. Because the MHV-A59 polymerase proteins have been difficult to identify during infection, we used a novel method to demonstrate the viral specificity of the antiserum. The viral cDNA was excised from the expression vector, and transferred to a pGem vector, downstream from and in-frame with a portion of the *cat* gene. This construct contained a bacteriophage RNA polymerase promoter that enabled the cell-free synthesis of a fusion protein that was used to verify that antibodies were generated to the expressed viral DNA. This strategy was shown to successfully result in the specific generation of antibodies to the encoded information of the viral cDNA. Furthermore, this method has general applicability in the generation and characterization of antibodies directed against proteins encoded in cDNAs.

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Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); βGal, β-galactosidase; B/V, bacterial/viral (fusion protein); CAT, Cm acetyl transferase; *cat*, gene encoding CAT; cDNA, DNA complementary to RNA; Cm, chloramphenicol; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; MHV, mouse hepatitis virus; moi, multiplicity of infection; NP40, Nonidet P40; nt, nucleotide(s); ONPG, *o*-nitrophenyl-D-galactopyranoside; ORF, open reading frame; PAGE, polyacrylamide-gel electrophoresis; PBS, 0.9% NaCl/10 mM Na·phosphate pH 7.4; PMSF, phenylmethylsulfonyl fluoride; RIPA buffer, 0.1% SDS/1% NP40/400 mM NaCl/25 μg PMSF per ml/20 μg aprotinin per ml/10 mM Na·phosphate pH 7.4; SDS, sodium dodecyl sulfate; TS, 10 mM Tris pH 7.4/10 mM NaCl/1.5 mM $MgCl_2$; TS/P, TS with 20 μg PMSF/ml; wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

INTRODUCTION

During infection of murine cells, coronavirus MHV-A59 generates at least four viral nonstructural proteins (Spaan et al., 1988). These proteins, present at low levels in infected cells, are difficult to detect against the background of cellular proteins. Some of these proteins have been detected in infected cells and in the products of cell-free translation of viral mRNAs (Leibowitz et al., 1982; 1988; Skinner et al., 1985). We have previously used a vector generating a tripartite protein containing RecA-viral-βGal aa sequences to generate antisera against a nonstructural protein encoded in gene *E* of MHV-A59 (Leibowitz et al., 1988). The MHV-A59 RNA-dependent RNA polymerase is presumed to be encoded in gene *A*, the approx. 23-kb sequence at the 5′ end of the genome RNA (Pachuk et al., 1989). Cell-free translation of genome RNA has allowed the identification of a group of proteins, 28- and 200- to 250-kDa (Denison and Perlman, 1986), which are probably encoded in the 5′ portion of gene *A* (Soe et al., 1987). The protein products of the rest of gene *A* have yet to be identified. We demonstrate here a method for the generation of antisera against the polypeptides encoded in the viral polymerase-encoding gene using a vector in which the viral segment is randomized by homopolymer addition (Ulrich et al., 1982) and inserted between a truncated *recA* and the *lacZ* gene, to generate B/V fusion proteins as immunogens. Unique to our work is a method to verify the viral specificity of these antisera using second fusion proteins. The viral segments are inserted into a second plasmid in which the *cat* gene is upstream from the viral sequences and provides the ATG for initiation of translation. RNA transcripts are generated in vitro from a T7 bacteriophage promoter and translated in a reticulocyte-free system. These viral/CAT fusion proteins were used to verify the viral specificity of the sera raised against the RecA/viral/βGal B/V proteins. In this way, we have been able to verify the viral specificities of the antisera without knowing the identity of the polypeptides encoded in this gene. This method of verification of the specificities of antisera will be applicable to other systems in which the protein product of an ORF has not been identified.

MATERIALS AND METHODS

(a) Eukaryotic cells and viral RNA

MHV-A59 was propagated in murine fibroblast 17Cl-1 cells. Viral genome was isolated from purified virions and cytoplasmic RNA was isolated from infected and mock-infected 17Cl-1 cells as described previously (Budzilowicz et al., 1985).

(b) Isolation of synthesized fusion proteins

For preparation of tripartite proteins for immunogens, bacterial cultures were prepared and induced with mitomycin C. Cells were harvested by centrifugation at 4°C and the pellet was resuspended in 20 ml of TS/P, and sonicated on ice in the presence of 2% Triton X-100 as described previously (Leibowitz et al., 1988). Lysozyme was added to 2 mg/ml, RNase A to 10 μg/ml, and DNase I to 1 μg/ml; the bacterial suspension was incubated at 37°C for 30 min, centrifuged for 30 min at 4°C at 10000 × *g* and the pellet was resuspended in TS/P-2% Triton X-100, washed three times and finally resuspended in 1–2 ml of TS/P and stored at −20°C (Leibowitz et al., 1988). βGal activity was monitored in the gross bacterial suspension and the supernatant and pellet fractions using the chromogenic substrate, ONPG.

(c) Generation of antibodies to fusion proteins

Purified fusion proteins isolated from the pellets described in section **b** above were used as immunogens in NZW rabbits as described by Leibowitz et al. (1988) except that each rabbit received 50 μg of aggregated fusion protein in one hind limb and 50 μg of denatured, reduced protein (1% SDS and 5% 2-mercaptoethanol, 100°C for 5 min) in the other limb. The development of an immune response to the βGal portion of the proteins was monitored by dot immunoassay (Leibowitz et al., 1988).

RESULTS AND DISCUSSION

(a) Rationale for the use of the recombinant ORF expression vector pGE374

Fig. 1A diagrams the ORF vectors used in our experiments. In the prokaryotic expression vectors, pGE372 and pGE374, the 5′ end of the *Escherichia coli recA* structural gene (35 codons), is located upstream from the *E. coli lacZ* gene, which lacks the promoter and translation start site. The *lacZ* gene encodes a functional βGal. However, synthesis of the enzyme requires the *recA* transcription and translation initiation signals. In pGE372, the *recA* and *lacZ* genes are in-frame. In pGE374, the downstream *lacZ* sequence is out-of-frame with the *recA* sequence. The *lacZ* and *recA* coding sequences are separated by a small synthetic DNA fragment; this region contains a *Sma*I site for insertion of DNA fragments. The *Sma* I site is flanked by two *Bam* HI sites, which are useful for the removal of the inserts. Fig. 1B shows the location on the viral genome of the relevant cDNAs.

Fig. 2 illustrates the construction of a pGE374/viral fusion vector using MHV A59 cDNA 1533. This 1.4-kb cDNA represents a portion of the 5′ region of MHV gene *A* (Fig. 1B), the putative viral polymerase-encoding gene. The recombinant plasmid will be referred to as pGE374/1533.

Fig. 3 shows the electrophoretic mobilities of proteins extracted from cells containing wt and recombinant pGE374/1533 plasmids. Coomassie blue

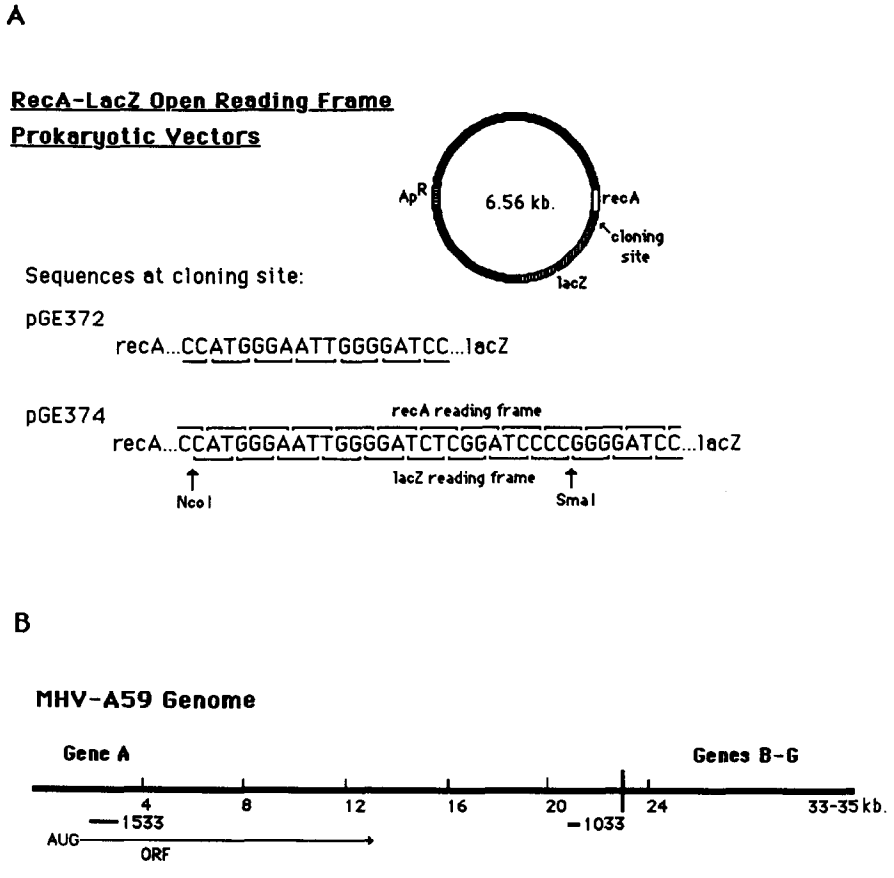

Fig. 1. Prokaryotic plasmid and MHV-A59 components of fusion vectors. (Panel **A**) Plasmids pGE372 and pGE374, pBR322 derivatives, which differ only at the cloning site between the *recA* and *lacZ* genes, which are in-frame in pGE372 and out-of-frame in pGE374 (Leibowitz et al., 1988). (Panel **B**) MHV-A59 genome and cDNAs. The 5′ 23 kb of genome are gene *A*, the putative polymerase-encoding gene. The 1033 and 1533 cDNAs, represented by short bars, were cloned from MHV-A59 genome (Gubler and Hoffman, 1983); the 1533 cDNA maps in the 5′ portion of gene *A*, while cDNA 1033 maps in the 3′ portion (Pachuk et al., 1989). The first ORF of gene *A* starts at nt 210 (Pachuk et al., 1989) and ends at approx. nt 13000. The vertical line shows the 3′ boundary of gene *A*; 3′ of gene *A* are the remaining MHV-A59 genes, *B-G*.

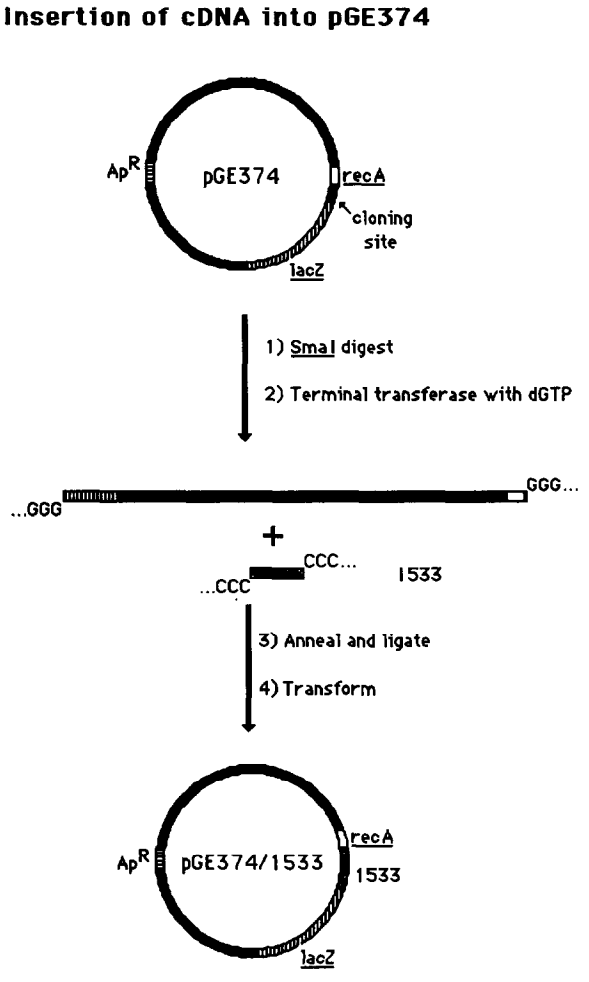

Fig. 2. Construction of *recA*/viral/*lacZ* B/V fusion vectors. Plasmid pGE374 (Fig. 1A) was linearized with *Sma*I and tailed with dGTP using terminal deoxynucleotidyl transferase (Deng and Wu, 1981). MHV-A59 cDNA, 1533, representing 1.4 kb of MHV-A59 gene *A* (Fig. 1B) was isolated from pBR322 by *Pst*I digestion, tailed with dCTP, annealed to *Sma*I digested, oligo(dG) tailed pGE374, and used to transform *E. coli* MC1061 (Dagert and Erhlich, 1979). Colonies expressing a LacZ$^+$ phenotype were selected in the presence of XGal. Plasmids were isolated from these colonies and digested with *Bam*HI, fractionated by agarose gel electrophoresis, transferred to nitrocellulose and probed with 1533 DNA (labeled with ^{32}P by nick translation (not shown) (Rigby et al., 1977; Southern, 1975). This confirmed that the plasmid contained virus-specific inserts.

staining shows that pGE372 generates a band of the size expected for the hybrid RecA/βGal protein (panel A, lane 2). Immunoblot analysis with anti-RecA serum (Fig. 3B, lane 2) confirms that this band is the RecA/βGal fusion protein. Extracts of cells bearing pGE374/1533 (Fig. 3A, lanes 3 and 4) contain a protein larger than the RecA/βGal hybrid

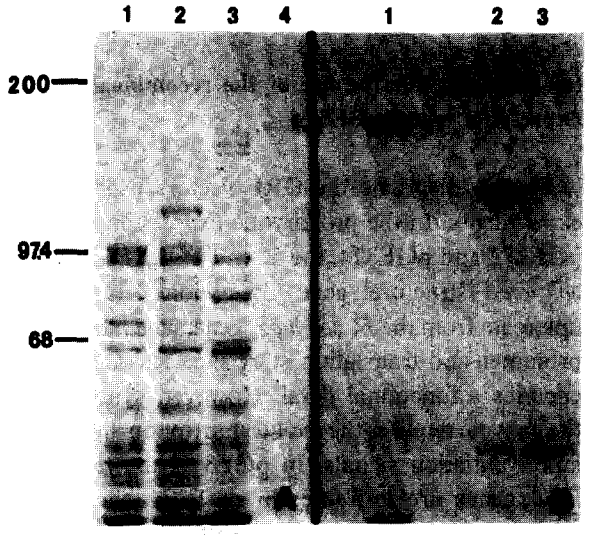

Fig. 3. Gel electrophoresis of B/V hybrid proteins. Mini-preps of pGE372, pGE374 and pGE374/1533 (Birnboim and Doly, 1979) were harvested by centrifugation and resuspended in TS, sonicated for three 20-s bursts, mixed with an equal volume of 2 × SDS-PAGE sample buffer (Maizel, 1971), and immersed in a boiling water bath for 5 min and clarified by centrifugation. Alternatively bacterial lysates were fractionated into pellet and supernatant fractions, as described in MATERIALS AND METHODS, section **b**, and proteins recovered from the pellet. Discontinuous SDS–PAGE was carried out (Maizel, 1971) with 8% resolving gels, and either stained with Coomassie blue (panel **A**) or used for Western immunoblotting (panel **B**) (Towbin et al., 1979). (Panel **A**) Coomassie-blue-stained gel of proteins from pGE374 (lane 1), pGE372 (lane 2), pGE374/1533 (lane 3), and pGE374/1533 proteins derived from the pellet after lysis (lane 4). The arrowhead indicates the tripartite B/V fusion protein in lane 4. (Panel **B**) Western-blot analysis of proteins from pGE374/1533 (lane 1), pGE372 (lane 2) and pGE374 (lane 3) using a polyclonal anti-RecA antibody. The arrowhead indicates the tripartite fusion protein in lane 1. Note the staining of the lower-molecular-weight RecA protein encoded in the bacterial chromosome as well.

which is not present in cells carrying pGE372 or pGE374. The fact that this protein contains RecA aa sequences (Fig. 3B, lane 1) is strong evidence that this protein is a RecA/viral/βGal B/V protein. The lower M_r RecA protein derived from the bacterial chromosome is also observed after staining with anti-RecA antibody (Fig. 3B, lanes 2 and 3).

The tripartite B/V fusion protein purification was based on the observation that when cells were lysed with lysozyme, Triton and sonication (MATERIALS AND METHODS, section **b**), the proteins are insoluble. The pellet derived from cell lysates contains mostly the B/V protein (Fig. 3A, lane 4). The rabbits were

inoculated with this aggregated protein, as well as with protein that had been reduced and denatured (MATERIALS AND METHODS, section c). After three to four boosts the rabbit antisera were shown to have activity against the tripartite B/V protein immunogen by a dot immunoassay (not shown) and were tested for reactivity with the viral protein sequences.

(b) Verification of antibodies generated to fusion proteins

To validate the production of antibodies to the expressed foreign DNA, a CAT/viral plasmid was constructed (Fig. 4). In the resulting plasmid, pGem4CAT/1533, the viral sequences are downstream from and in-frame with a truncated *cat* gene, which contains an ATG for protein synthesis initiation and is in turn downstream from a bacteriophage T7 RNA polymerase promoter. A control vector, pGem4CAT/372, was constructed using an *Nco*I-*Pvu*II fragment from pGE372.

Synthetic RNAs transcribed from pGem4CAT/1533 and pGem4CAT/372 were translated in a reticulocyte-free system (Fig. 5). Translation of the CAT/1533 RNA results in two major proteins. The larger 74-kDa polypeptide is the size expected for a CAT/1533 protein which would contain part of the N terminus of the CAT protein, 27 aa from the N terminus of βGal, as well as the coding information in the 1533 insert (approx. 450 aa). The smaller protein is the size expected for the CAT protein terminating at the *recA*/viral junction, probably due to the homopolymer sequence resulting from tailing. The major product of translation of the control RNA is a 24-kDa protein consistent with the size of the CAT/372 sequence. These translation products were immunoprecipitated with antiserum raised against the RecA/1533/Gal protein, to verify the viral specificity of the antiserum. The CAT/372 protein was a necessary control for the small amount of βGal still present in the CAT/1533 hybrid protein. As shown in Fig. 5, antibodies to the RecA/1533/ βGal fusion

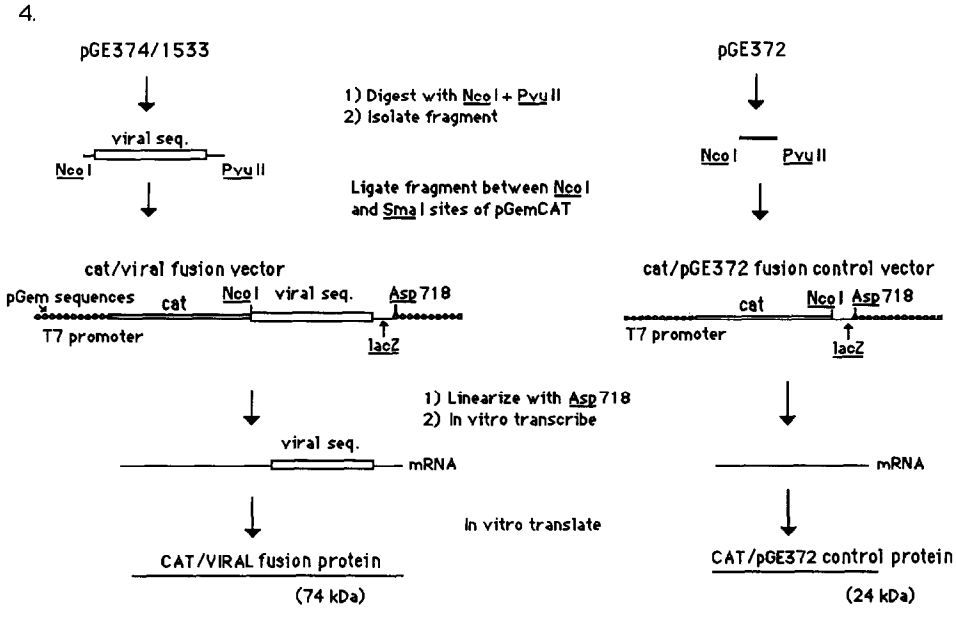

Fig. 4. Use of viral CAT/viral fusion proteins to determine the specificity of antiserum. The pGem4CAT vector was constructed by inserting 550 bp of the 5′ end of the *cat* gene (including the ATG start codon), derived as a *Hin*dIII-*Ban*I fragment, from the pRSV2CAT plasmid (provided by Dr. J. Alwine) into *Hin*dIII + *Pst*I cleaved pGem4 downstream from a bacteriophage T7 RNA polymerase promoter. The 1533 viral sequences were excised from pGE374/1533 with *Nco*I (at the 5′ end of the *recA* gene) and *Pvu*II (in the *lacZ* gene) and inserted into pGem4CAT downstream from the *cat* sequences and the T7 bacteriophage promoter. This fragment contained, in addition to the viral sequences, 82 nt of the *lacZ* gene. A control vector was constructed by insertion of the small *Nco*I-*Pvu*II fragment of pGE372 into pGem4CAT. These recombinant vectors were linearized with *Asp*718 and transcribed using the bacteriophage T7 RNA polymerase in the presence of 500 mM ^{7}G(5′)ppp(5′)G. The template was removed with DNase I and the RNA recovered all as described by Krieg and Melton (1984). RNAs (1 μg) were translated in 25 μl of rabbit reticulocyte lysate (Promega Biotec) containing 10 units of RNasin, 25 μg of soybean trypsin inhibitor, 10 μg of leupeptin, and 10 μCi of [^{35}S]methionine. Following a 1-h incubation at 31°C, the reaction was terminated by dilution into RIPA buffer.

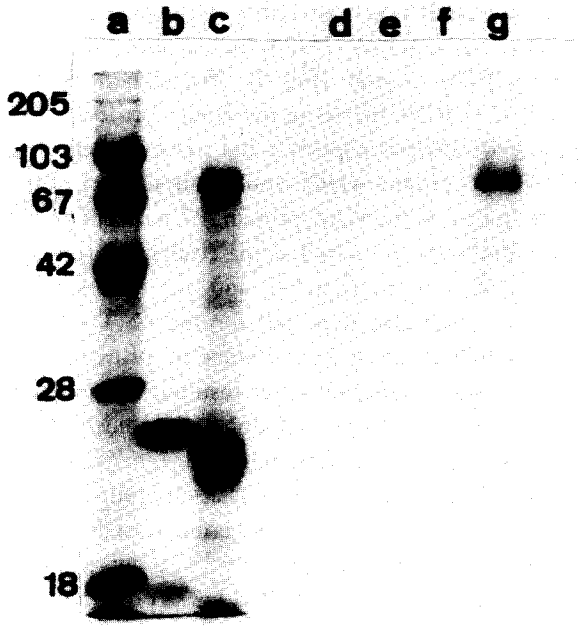

Fig. 5. Immunoprecipitation of CAT/viral protein by 1533 antiserum. The viral/CAT fusion protein and the control pGE372 protein, synthesized as described in Fig. 4, were immunoprecipitated with pre-immune and 1533 antisera which had been concentrated by precipitation in the presence of 40% ammonium sulfate. Immunoprecipitations were carried out as described previously (Leibowitz et al., 1988) and the resulting proteins were analyzed by SDS-7.5%PAGE (Maizel, 1971). Lanes: a, protein M_r markers (sizes in kDa in the lefthand margin); b, control protein analyzed directly; c, viral/CAT protein analyzed directly; d, control protein immunoprecipitated with pre-immune serum; e, viral/CAT protein immunoprecipitated with pre-immune serum; f, control protein immunoprecipitated with 1533 serum; g, viral/CAT protein immunoprecipitated with 1533 serum. There are no bands observed in lanes d–f.

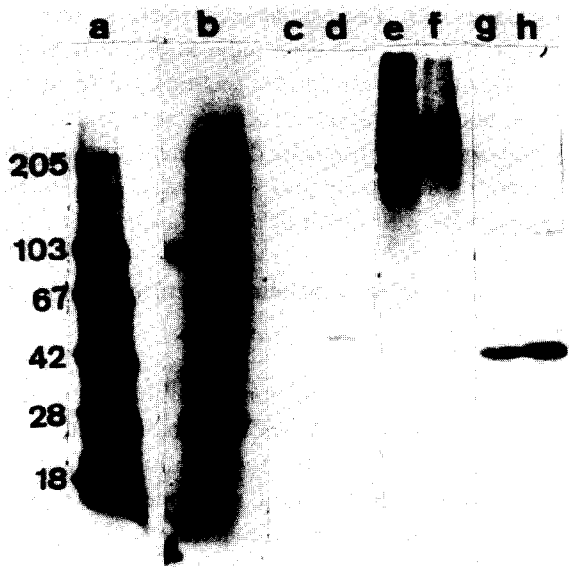

Fig. 6. Immunoprecipitation of cell-free translation products of genome RNA by 1533 antiserum. MHV-A59 genome RNA was translated in a rabbit reticulocyte-free system and either analyzed directly or after immunoprecipitation on SDS-(4–15%)PA gradient gels as in Fig. 5. Lanes: a, protein M_r markers; b, products analyzed directly; c–h, products immunoprecipitated; c, pre-immune serum; d, anti-MHV-A59 nucleocapsid monoclonal antibody; e, 1533 (rabbit A) antiserum; f, 1533 (rabbit B) antiserum; g, 1033 (rabbit A) antiserum; h, 1033 (rabbit B) antiserum.

protein immunoprecipitated the in vitro translation product of pGem4CAT/1533 RNA but not the control pGEMCAT/372. Thus, the antiserum must contain antibodies which recognize the viral sequences. The fact that the smaller protein derived from the CAT/1533 fusion does not immunoprecipitate, is consistent with our hypothesis that this protein contains CAT sequences but not viral sequences.

To further verify its viral specificity, the antiserum was used to immunoprecipitate the in vitro translation products of the MHV-A59 genome RNA (Fig. 6). In vitro translation of the viral genome results in several related proteins of 200–250 kDa (Leibowitz et al., 1982; Denison and Perlman, 1986) that correspond to the ORF initiated at the 5′ end of the genome, the putative viral polymerase-encoding gene (Soe et al., 1987). Since the 1533 cDNA contains sequences found approx. 5.3–6.7 kb from the 5′ end of the viral genome (Pachuk et al., 1989) (Fig. 1B), antiserum directed against the sequences encoded in this cDNA should react with the products of in vitro translation of genome RNA. This indeed was the case. The antisera generated against the product of pGE374/1533 immunoprecipitate the group of large polypeptides synthesized from the rabbit reticulocyte-free system using virion RNA as template (Fig. 6). Antisera made against the proteins encoded in cDNAs more 3′ to 1533 within the gene *A* (such as cDNA 1033, Fig. 1B) did not immunoprecipitate these proteins (Fig. 6).

(c) Conclusions

The pGE374 plasmid has several features that make it an excellent expression vector. (1) The strategy for insertion of foreign DNA into pGE374

requires no detailed sequence information to determine if a coding sequence is present in the fusion DNA. Statistically, if there are n ORFs out of the six possible ORFs for the insert, the ratio of LacZ$^+$ transformants to the total number of transformants is $n/18$. If there are N codons in a random piece of DNA, then the probability that in either polarity a contiguous ORF is present, is $2 \times (61/64)^N$. If N is greater than 110, there is less than a 1% chance that the ORF is the result of random occurrence. The 1533 cDNA (1.4 kb), inserted into the pGE374 vector resulted in a LacZ$^+$ phenotype in approx. 5% of transformants, suggesting it contains a single ORF. (2) The vector includes the *recA* regulatory elements necessary for high level transcription and translation of fusion proteins. Multiple copies of the vector and induction of the operon in the presence of mitomycin C contribute to the high expression. Identification of both the N-terminal RecA sequences and the C-terminal βGal sequences are easily accomplished (RESULTS AND DISCUSSION, section **a**; Fig. 3). This and the relative insolubility of the fusion protein make purification simple. (3) Because this system can be used for expression of relatively large fragments of DNA, the fusion protein is likely to display sequential and conformational epitopes when used as an immunogen.

The 200- to 250-kDa products of the 23-kb MHV-A59 putative polymerase-encoding gene have not been identified in the infected cell probably due to low abundance of these nonstructural products and the lack of efficient suppression of host-cell protein synthesis during infection. Therefore, the only assay for the sera available is the reaction with the products of cell-free translation of genome RNA, in which the first 7–8 kb of genome are translated (Denison and Perlman, 1986). To verify the viral specificity of antisera made against polypeptides encoded in more 3′ portions of the polymerase-encoding gene, the *cat*/viral plasmids were constructed. In vitro translation of RNA from the *cat*/1533 vector was used to demonstrate that the 1533 antiserum did contain virus-specific antibodies. This was verified by immunoprecipitation of the products of cell free translation of genome RNA (Fig. 5). We have now demonstrated that this antiserum specifically stains infected cells by an immunofluorescence assay and immunoprecipitates several large proteins from infected cells (P.W.Z., J.L.L. and S.R.W., manuscript in preparation). This CAT/viral fusion protein assay will be even more important for antisera derived against proteins encoded further downstream in the putative polymerase-encoding gene (for example, 1033, see Fig. 1B), in which synthesis of protein from the genomic template in a cell-free translation system is not observed.

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