JOURNAL OF VIROLOGY, Jan. 1983, p. 462–465
0022-538X/83/010462-04$02.00/0

Vol. 45, No. 1

Development of Anti-pp60^{src} Serum with Antigen Produced in *Escherichia coli*

TONA M. GILMER AND R. L. ERIKSON*

Department of Pathology, University of Colorado School of Medicine, Denver, Colorado 80262

Received 12 July 1982/Accepted 30 September 1982

We have purified p60^{src} from bacterial recombinants which direct the synthesis of the Rous sarcoma virus transforming gene (*src*) product. This protein was injected into rabbits, and they produced a highly cross-reactive serum which can recognize the *src* protein from many different strains of Rous sarcoma virus.

Rous sarcoma virus (RSV) is responsible for cellular transformation as well as tumor formation in a variety of animals (11, 13). The product of the RSV transforming gene, *src*, was first identified by the use of antisera from rabbits that had developed tumors after being injected with RSV as newborns (2). Tumor-bearing rabbit (TBR) sera recognize a phosphoprotein of molecular weight (M_r) 60,000 (pp60^{src}) from extracts of RSV-infected avian or mammalian cells (2). Most antisera obtained from tumor-bearing rabbits are reactive only with the pp60^{src} encoded by the Schmidt-Ruppin strain of RSV, the strain used to produce the tumors. A few TBR sera also precipitate the pp60^{src} encoded by other strains of RSV, and a small number also immunoprecipitate a M_r = 60,000 phosphoprotein from uninfected cells, designated pp60$^{c\text{-}src}$ (6). Antiserum prepared in this manner requires the purification of large amounts of virus which must be injected into newborn rabbits. The time during which high-titer antibody to pp60^{src} is expressed is relatively short, and the amount of antiserum obtained is small because the rabbits are only several weeks old. Recombinant DNA techniques have made possible the production of large amounts of p60^{src} (9, 10), and in this paper we describe the use of p60^{src} expressed in *Escherichia coli* as an antigen to circumvent some of these problems.

Bacteria containing a *lac-src* plasmid were grown in M9 medium (1) plus 1 mM isopropyl-β-D-thiogalactopyranoside to an absorbance at 550 nm of 1.2, and lysates were prepared as described previously (10). After clarification of the lysates at 16,000 × *g* for 30 min, 90% of the p60^{src} was in the pellet. The pellet was washed once with 1% Nonidet P-40 (NP-40)–0.5% sodium deoxycholate–100 mM NaCl–10 mM Tris (pH 7.2)–1 mM EDTA, once with 1 M NaCl–0.1% NP-40–10 mM Tris (pH 7.2), and three times with 1% Triton X-100–1% sodium deoxycholate–150 mM NaCl–10 mM Tris (pH 7.2)–0.1% sodium dodecyl sulfate (SDS)–1 M urea. The pellet was suspended in sample buffer (70 mM Tris [pH 6.8]–11.2% glycerol–3% SDS–0.002% bromophenol blue–5% β-mercaptoethanol), incubated at room temperature for 15 min, and subjected to polyacrylamide gel electrophoresis. Proteins were visualized by staining with 0.2% Coomassie blue–50% methanol–12% glacial acetic acid. After extensive washing with water, the band containing p60^{src}, approximately 40 μg of protein, was excised from the gel. The protein was eluted in phosphate-buffered saline overnight, complete Freund adjuvant was added, and the emulsion was injected into the popliteal lymph nodes of two rabbits. The remaining gel piece was homogenized, complete Freund adjuvant was added, and the suspension was injected subcutaneously and intramuscularly into the two rabbits. For the first boost, approximately 200 μg of p60^{src} was prepared in the same way, except that the pellet was heated to 95°C for 1 min in sample buffer before electrophoresis. The entire gel piece containing p60^{src} was homogenized in incomplete Freund adjuvant and injected intramuscularly, 16 days after the initial injection.

Protein for subsequent boosts, which were administered 1 and 2 months after the initial injection, was prepared as described above through the washes of the 16,000 × *g* pellet. At that point, the pellet was solubilized in 9.5 M urea–5% β-mercaptoethanol and incubated at 37°C for 15 min. The urea was diluted to 6 M, and potassium phosphate (pH 7.2) was added to a final concentration of 20 mM. This sample was mixed with an identical preparation from bacteria which had been labeled with $^{35}SO_4^{2-}$ (9) and was applied to a hydroxyapatite column equilibrated in the same buffer. The column was

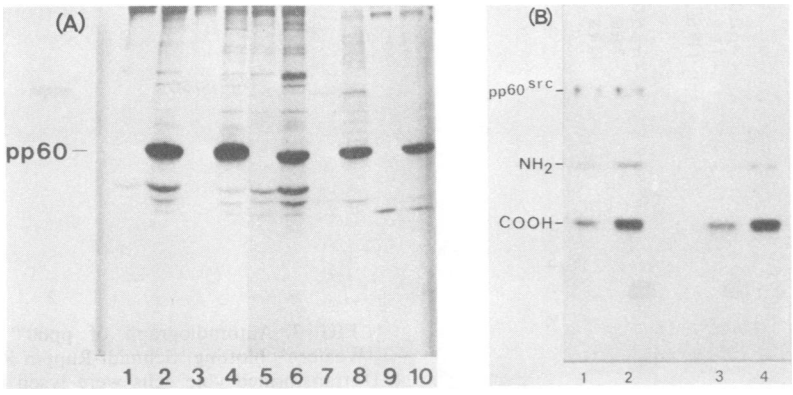

FIG. 1. (A) Immunoprecipitation of pp60^{src} from RSV-transformed cells. Cells were labeled with [^{35}S]methionine as described previously (2). After immunoprecipitation of extracts with either preimmune serum (lanes 1, 3, 5, 7, 9) or anti-p60 serum (lanes 2, 4, 6, 8, 10), the samples were electrophoresed in SDS-polyacrylamide gels (12) and autoradiographed. Lanes 1 and 2, CEF infected with Prague strain of RSV, subgroup A (PrA-RSV); lanes 3 and 4, CEFs infected with Prague strain of RSV, subgroup C; lanes 5 and 6, CEFs infected with Schmidt-Ruppin RSV, subgroup A; lanes 7 and 8, vole cells transformed by Schmidt-Ruppin RSV, subgroup D; lanes 9 and 10, rat kidney cells transformed by Bratislava 77 strain of RSV. (B) Partial proteolytic cleavage of the M_r = 60,000 protein. The M_r = 60,000 protein was immunoprecipitated from an extract of [^{35}S]methionine-labeled PrA-RSV-transformed CEFs as shown in Fig. 1A, lane 2. In addition, pp60^{src} was immunoprecipitated from the same cells by TBR serum. The two M_r = 60,000 proteins were excised from preparative polyacrylamide gels and subjected to partial proteolysis during re-electrophoresis as described by Cleveland et al. (4). Lanes 1 and 3, pp60^{src}; lanes 2 and 4, M_r = 60,000 protein; lanes 1 and 2, digestion with 5 ng of *Staphylococcus aureus* V8 protease; lanes 3 and 4, 50 ng of *S. aureus* V8 protease. The two large fragments of pp60^{src}, which are the amino and carboxy termini, are designated NH_2 and COOH, respectively (7).

washed with 3 volumes of the loading buffer followed by 3 volumes of 6 M urea–100 mM potassium phosphate (pH 7.2)–50 mM β-mercaptoethanol and then was eluted with 6 M urea–400 mM potassium phosphate (pH 7.2)–50 mM β-mercaptoethanol. Fractions containing the peak of ^{35}S counts were pooled and dialyzed against 50% glycerol containing 1 mM EDTA–20 mM potassium phosphate (pH 7.2)–5 mM β-mercaptoethanol followed by dialysis against 10% glycerol in the same buffer. A sample of this preparation was analyzed by SDS-polyacrylamide gel electrophoresis and was shown to consist mostly of p60^{src} by Coomassie blue staining (data not shown). The protein concentration of the final purified product was about 250 μg/ml. The suspension was diluted to 5% glycerol with phosphate-buffered saline, incomplete Freund adjuvant was added, and 100 μg of protein was injected intramuscularly into each of the two rabbits.

Sera obtained from the rabbits were assayed by immunoprecipitation of extracts of [^{35}S]methionine-labeled RSV-infected cells (2). Both antisera immunoprecipitated a protein (M_r = 60,000) from various cells transformed by any of several strains of RSV (Fig. 1A). Preimmune sera did not immunoprecipitate such a protein. To confirm that the M_r = 60,000 protein precipitated by these antisera was indeed pp60^{src}, we compared the partial proteolytic cleavage map of this protein with that of pp60^{src} immunoprecipitated with TBR serum. The two maps were identical (Fig. 1B), and so these sera will be referred to as anti-p60 serum. A low titer of anti-pp60^{src} activity was detectable in the serum from one rabbit on day 16. One month after the initial injection, sera from both rabbits were weakly positive. The titers of both sera increased until 10 weeks after the initial injection and remained high for approximately 4 more weeks. The titers then decreased considerably, and additional boosts failed to elicit an increased response. The serum used for the analyses presented in this communication was obtained at 10 to 12 weeks. Since the titers of TBR sera are quite variable, it is difficult to compare the activity of anti-p60 serum with that of TBR serum. However, when assayed in antigen excess, these sera immunoprecipitated approximately 30 to 40% more Prague strain subgroup A (PrA) pp60^{src} than did a high-titer TBR serum available in this laboratory at the present time.

Anti-p60 sera were also tested for their capacity to immunoprecipitate denatured pp60^{src}. Anti-p60^{src} serum immunoprecipitated SDS-denatured p60^{src} from either p60^{src}-producing *E. coli* or from Schmidt-Ruppin RSV (subgroup D)-infected vole cells (Fig. 2). In contrast, TBR serum did not immunoprecipitate denatured pro-

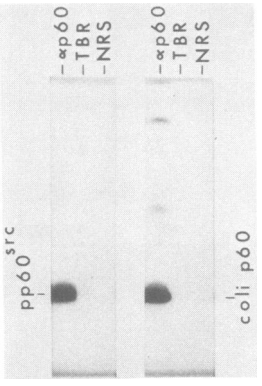

FIG. 2. Immunoprecipitation of denatured pp60^{src}. A preparation of pp60^{src} from eucaryotic cells was phosphorylated by incubation with 5 mM Mg^{2+} and 1 μM [γ-^{32}P]ATP (8), and a preparation of p60^{src} from *E. coli* containing *lac-src* plasmids was phosphorylated by incubation with 5 mM Mg^{2+}, 1 μM ^{32}P]ATP, and the catalytic subunit of cyclic AMP-dependent protein kinase (9). The products of the reaction were resolved by SDS-polyacrylamide electrophoresis, and the p60 bands were visualized by autoradiography and eluted from the gel slices by incubation at 37°C overnight with 0.05 M NH_4HCO_3 (pH 8.5)–0.1% SDS. The eluates were adjusted to 1% NP-40–0.5% sodium deoxycholate–100 mM NaCl–10 mM Tris (pH 7.2)–1 mM EDTA and then were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis as indicated.

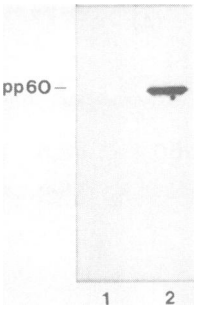

FIG. 3. Autoradiograph of pp60^{src} detected by "Western" blotting. Schmidt-Ruppin RSV (subgroup D)-transformed vole cells were lysed in 1% NP-40–0.05% sodium deoxycholate–100 mM NaCl–10 mM Tris-hydrochloride (pH 7.2)–1 mM EDTA and clarified by centrifugation at 100,000 × *g* for 30 min at 4°C. Samples of the supernatant fraction containing 100 μg of protein were adjusted to 1× sample buffer and electrophoresed on a 10% SDS-polyacrylamide gel. Proteins were electrophoretically transferred (3) from the gel to a sheet of nitrocellulose (BA 85; Schleicher & Schuell Co.). After transfer, the nitrocellulose was incubated at 4°C overnight and then at 40°C for 1 h in 10 ml of 10 mM Tris-hydrochloride (pH 7.4)–150 mM NaCl–0.02% sodium azide–3% bovine serum albumin (BSA fraction V; Miles Laboratories, Inc.) to block the unreacted protein binding sites. The sheet was rinsed in water and divided into two parts. The sheets were then incubated at room temperature for 2 h with either preimmune serum or anti-p60 serum in the saturation buffer described above with 1% fetal calf serum. The sheets were rinsed in water and incubated at room temperature for 2 h with ^{125}I-labeled protein A (10^6 cpm/ml) in 3 ml of 10 mM Tris-hydrochloride (pH 7.4)–150 mM NaCl–0.05% Triton X-100 (saline buffer). The sheets were washed once with 1 M NaCl–0.1% NP-40–10 mM Tris-hydrochloride (pH 7.2), twice with 1% Triton X-100–1% sodium deoxycholate–150 mM NaCl–10 mM Tris-hydrochloride (pH 7.2)–0.1% SDS–1 M urea, once with saline buffer, and once with water. After drying, the sheets were autoradiographed. Lane 1, preimmune serum; lane 2, anti-p60 serum.

tein (Fig. 2). This property indicated that the anti-p60 sera would be useful in the "Western" (protein) blotting technique (3), and the results of those analyses are shown in Fig. 3.

When pp60^{src} was immunoprecipitated with anti-p60 serum from PrA-RSV-infected chicken embryo fibroblasts (CEFs) and [γ-^{32}P]ATP was added to the immune complex (7), we failed to detect any transfer of P_i to the heavy chain of immunoglobulin G (IgG). In this respect, the antiserum is similar to sera from RSV tumor-bearing mice, sera which also cannot be recognized as substrates in the immune complex kinase assay (6). It has been previously shown that in soluble phosphotransferase reactions, TBR IgG inhibits the phosphorylation by pp60^{src} of exogenous substrates such as casein (8, 9). Anti-p60 IgG also inhibits the phosphorylation by pp60^{src} of exogenous substrates (Y. Graziani, unpublished data).

The availability of large amounts of *src* protein has made possible the production of high-titer antisera that are cross-reactive with pp60^{src} from a wide variety of strains of RSV. Because these antisera do not immunoprecipitate viral structural proteins, they will be particularly useful in determining the relatedness of other sarcoma virus transforming gene products.

We thank G. Miller for injecting the rabbits and for helpful discussions. We are grateful to N. Fishman for excellent technical assistance and to R. H. Cook-Deegan for help with the "Western" blotting analysis.

T.M.G. was supported by postdoctoral fellowship grant CA 06839 from the National Institutes of Health (NIH). This work was supported by grants CA-21117 and CA-15823 from NIH and by an award from the American Business Cancer Research Foundation and, indirectly, by a gift from the R. J. Reynolds Industries, Inc., to the Department of Pathology.

LITERATURE CITED

1. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68**:245–267.
2. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–347.
3. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-poly-

acrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.

4. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
5. **Collett, M. S., E. Erikson, and R. L. Erikson.** 1979. Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. J. Virol. **29**:770–781.
6. **Collett, M. S., E. Erikson, A. F. Purchio, J. S. Brugge, and R. L. Erikson.** 1979. A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product. Proc. Natl. Acad. Sci. U.S.A. **76**:3159–3163.
7. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2021–2024.
8. **Erikson, R. L., M. S. Collett, E. Erikson, A. F. Purchio, and J. S. Brugge.** 1979. Protein phosphorylation mediated by partially purified avian sarcoma virus transforming gene product. Cold Spring Harbor Symp. Quant. Biol. **44**:907–917.
9. **Gilmer, T. M., and R. L. Erikson.** 1981. Rous sarcoma virus transforming protein, $p60^{src}$, expressed in *E. coli*, functions as a protein kinase. Nature (London) **294**:771–773.
10. **Gilmer, T. M., J. T. Parsons, and R. L. Erikson.** 1982. Construction of plasmids for expression of Rous sarcoma virus transforming protein, $p60^{src}$, in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **79**:2152–2156.
11. **Hanafusa, H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Publishing Corp., New York.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Vogt, P. K.** 1977. The genetics of RNA tumor viruses, p. 341–455. *In* H. Fraenkel-Conrat and R. Wagner (ed.), Comprehensive virology, vol. 9. Plenum Publishing Corp., New York.