JOURNAL OF VIROLOGY, May 1983, p. 594–605
0022-538X/83/050594-12$02.00/0

Vol. 46, No. 2

Construction and Isolation of a Transforming Murine Retrovirus Containing the *src* Gene of Rous Sarcoma Virus

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Received 22 November 1982/Accepted 14 February 1983

Recombinant murine retroviruses containing the *src* gene of the avian retrovirus Rous sarcoma virus were isolated. Such viruses were isolated from cells after transfection with DNAs in which the *src* gene was inserted into the genome of the amphotropic murine retrovirus 4070A. The isolated viruses had functional *gag* and *pol* genes, but they were all *env* defective since the *src* gene was inserted in the middle of the *env* gene coding region. Infectious transforming virus could be isolated only from cells transfected with DNA constructions in which the *src* gene was in the same polarity as that of a long terminal repeat of the amphotropic viral genome. These recombinant viruses encoded a $pp60^{src}$ protein with a molecular weight similar to that of the Schmidt-Ruppin strain of Rous sarcoma virus. In addition, the *src* protein(s) of these recombinant viruses was as active as protein kinases in the immune complex protein kinase assay. Intravenous injection of helper-independent Moloney and Friend murine leukemia virus pseudotypes of the *src* recombinant viruses into 6-week-old NIH Swiss mice resulted in the appearance of splenic foci within 2 weeks, splenomegaly and, later after infection (8 to 10 weeks), anemia. Infectious transforming virus could be recovered from the spleens of diseased animals. Such viruses encoded $pp60^{src}$ but not $p21^{ras}$ or mink cell focus-forming virus-related glycoproteins.

Since its isolation in 1911 by Peyton Rous (50), Rous sarcoma virus (RSV) has been an extremely important system for virus oncologists. RSV induces morphological transformation of chicken embryo fibroblasts in vitro (24) and causes fibrosarcomas after injection of chickens. The ability of RSV to induce tumors and morphological transformation has been correlated with the presence of the *src* gene (24, 68). The *src* gene encodes a tyrosine-specific protein kinase with a molecular weight of 60,000 (10, 11, 16, 36).

The ability of RSV to induce tumors in mammals and to cause transformation of mammalian cells has been extensively studied. Tumor formation generally occurs only after a long latency (57, 67, 74). The ability of different strains to induce tumor formation seems to correlate with the subgroup of the envelope glycoproteins of these strains rather than to intrinsic differences in the *src* genes of these isolates (8, 72). Furthermore, it is extremely difficult to recover infectious RSV directly from mammalian tumor material (1–3, 61, 62, 73) or from transformed mammalian cells (20, 56, 63). In most cases, efficient rescue of infectious virus can be accomplished only by cocultivating the transformed mammalian cells with chicken cells (56, 59, 63). The inability to rescue infectious virus is probably due to the fact that not all the viral genes are transcribed in RSV-transformed mammalian cells and that the level of viral RNA is reduced compared with that in virus-infected chicken cells (7, 12, 66). Furthermore, there seems to be a block in the processing of at least the *gag* proteins in nonavian cells (69).

With the advent of molecular cloning of retroviral genomes, it has become possible to construct recombinant viruses containing a variety of sequences, such as the thymidine kinase gene of herpes simplex virus (55, 64, 71). We have sought to construct a replication-defective transforming murine retrovirus which contains the *src* gene of RSV. Such a recombinant murine retrovirus would be of great interest to tumor virologists since it would be readily able to infect (and transform) the cells present in a wide variety of mammalian systems currently available to biologists. We report here the isolation of such a recombinant virus and note the difference between *src*-containing murine retroviruses and avian RSV.

MATERIALS AND METHODS

Cells. NIH 3T3 cells, a line of NIH 3T3 cells transformed by the Schmidt-Ruppin strain of RSV, a line

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of NRK cells nonproductively infected by the Friend spleen focus-forming virus, and a line of NIH 3T3 cells nonproductively transformed by Harvey murine sarcoma virus were obtained from the stocks of this laboratory as previously described (21, 39, 40, 51). They were all grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

DNA transfections. DNA transfections were performed by a modification of the method of Graham and van der Eb (22) as previously described (39, 40, 71). Briefly, 3×10^5 NIH 3T3 cells were seeded into 35-mm petri dishes and transfected 18 to 24 h later with 1 μg of helper viral DNA and 0.3 μg of DNA of the *src*-recombinant plasmid per plate. Plasmids 63-2, 63-6, 63-7, and 63-13 were not cut before transfection. The AMT8 and pSRC2 plasmids were cut with *Eco*RI before transfection. The cells were split 1:2 when the cultures reached confluence, and the supernatant fluids were assayed weekly for the presence of the polymerase-positive virus particles.

Construction of *src*-recombinants. The AMT8 plasmid was obtained from A. Oliff, Memorial Sloan-Kettering Cancer Institute, New York, N.Y. The pSRC2 plasmid, the kind gift of J. M. Bishop, University of California, San Francisco, contains a 3-kilobase (kb) *Eco*RI fragment from the Schmidt-Ruppin A strain of RSV (SRA-RSV). A partial digest of AMT8 DNA with *Eco*RI was prepared. DNA which had been cut at one *Eco*RI site was isolated by electrophoresis in 1.0% Sea-Kem agarose gels (FMC Corp., Marine Colloids Div., Rockland, Maine), and the DNA was removed from the gel by electroelution. The 3-kb *Eco*RI fragment from pSRC2 was isolated in the same fashion. The DNA fragments were ligated with T4 DNA ligase (New England Biolabs) according to the recommendations of the manufacturer. The reaction was carried out for 18 h at 4°C. The ligated DNAs were used to transform *Escherichia coli* RR1. Transformation was carried out by the method of Cohen et al. (15). Colonies were screened by the method of Grunstein and Hogness (23). The filters were baked for 2 h at 80°C in vacuo and hybridized to an *src*-specific probe. Hybridization was carried out at 42°C in 50% formamide, 5× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), and 0.1% sodium dodecyl sulfate (SDS). *src*-containing colonies were amplified and examined for the presence of *src* and amphotropic murine leukemia virus (MuLV) DNA sequences, and the arrangement of these sequences was determined. All DNA probes were prepared by nick translation (38). DNA blotting from 1.0% agarose gels to nitrocellulose filters (Schleicher & Schuell Co.) was performed by the technique of Southern (60). Hybridizations were performed as described above. All enzymes were obtained from Bethesda Research Laboratories, Inc.

Metabolic labeling of cells, immunoprecipitation, and SDS-gel electrophoresis. Labeling of cells was done as previously described (6). Plates (100 mm) of cells were starved in methionine-free minimal essential medium for 30 min, labeled for 4 h with 200 μCi of [^{35}S]-methionine (New England Nuclear) in 2 ml of methionine-free minimal essential medium, rinsed with phosphate-buffered saline, and then lysed in 1.0 ml RIPA buffer (150 mM NaCl, 50 M Tris [pH 7.4], 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.5% SDS with 1% Kallikrein Inactivator [Calbiochem]). Lysates were disrupted by vortexing for 30 s and spun at 36,000 rpm in a Beckman type 40 rotor for 20 min. Lysate (100 μl) and antibody (5 μl) were incubated on ice for 1 h, and then 15 μl of a 50% suspension of protein A-Sepharose (Pharmacia Fine Chemicals, Inc.) was added and the reaction was mixed on a rotating platform for 1 h at 4°C. Immune complexes were washed five times with RIPA buffer and analyzed on 10% SDS-polyacrylamide gels prepared according to the method of Laemmli (34). Gels were treated with En^{3}Hance (New England Nuclear), dried, and exposed to X-ray film at −70°C.

Immune complex protein kinase assay. The immune complex protein kinase assay was performed as described previously (6). The reaction buffer consisted of 60 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid [pH 7.0]), 0.15 M KCl, 5 mM dithiothreitol, 10 mM magnesium acetate, and 0.1 μM [^{32}P]ATP (3,000 Ci/mmol; Amersham Corp.). The reaction products were analyzed on 7.5% SDS-polyacrylamide gels.

Antiserum. Tumor-bearing rabbit serum was the gift of H. Opperman, University of California, San Francisco, Calif., and H. Hanafusa, Rockefeller University, New York. These sera recognize pp60^{src} and a few structural proteins characteristic of avian retroviruses (10). Antisera specific for MuLV p15, Rauscher MuLV gp70, and the gp70 of mink cell focus-forming viruses (MCFs) were provided by S. Ruscetti, National Cancer Institute, Bethesda, Md. M. Furth, National Cancer Institute, kindly provided monoclonal 259, which recognizes p21 of Harvey and Kristen sarcoma viruses (21). Rabbit anti-avian leukosis virus *gag* and *env* serum was produced as described previously (6).

Mice. Six-week-old female NIH Swiss mice were obtained from the National Institutes of Health, Bethesda, Md.

RESULTS

Construction of clones containing *src* and amphotropic viral sequences. The DNAs used in this study are shown in Fig. 1. The amphotropic virus 4070A was molecularly cloned in pBR322 at the *Eco*RI site within the *env* gene, and this clone, AMT8, contains a single long terminal repeat (LTR). The *src* gene was present in a 3-kb *Eco*RI fragment derived from the molecular clone of SRA-RSV (17). In addition to the *src* coding region, this DNA includes the coding region of SRA-RSV gp37, the direct repeats which flank the *src* gene, and the U3 region of the LTR (17, 71a). A partial digest of AMT8 DNA with *Eco*RI was prepared, and DNA which had been cut at only one of the two *Eco*RI sites was recovered. This DNA was ligated to the insert of pSRC2 which had been released by complete digestion with *Eco*RI, and the ligated DNAs were used to transform *E. coli* RR1. Colonies were screened for the presence of *src* sequences by colony hybridization. Clones which contained *src* sequences were amplified and characterized for the presence of both *src* and amphotropic viral sequences.

Clones representing the four possible arrange-

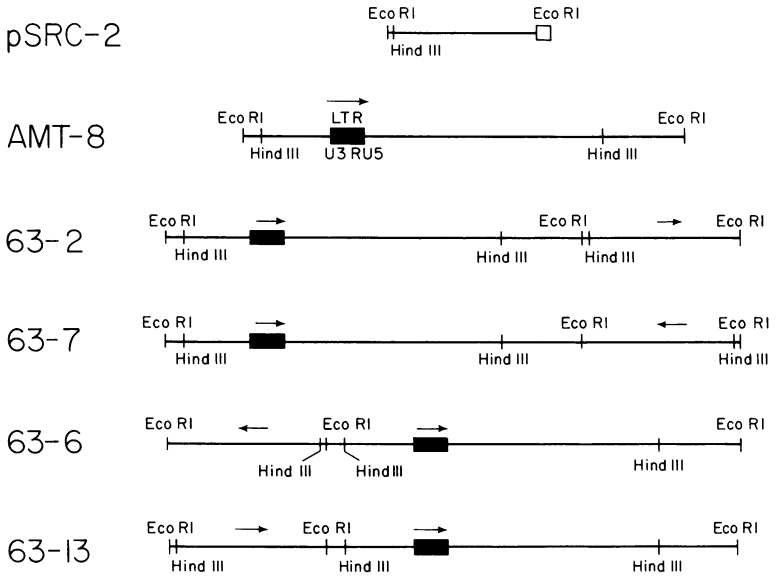

FIG. 1. Structure of DNA constructions used in this study. pSRC 2 is the 3-kb *Eco*RI fragment from SRA-RSV (17). This fragment contains the *env* sequences encoding gp37, the *src* gene, and the U3 region of the LTR (Schwartz et al., submitted for publication). AMT-8 is the amphotropic murine retrovirus 4070A which was molecularly cloned at a single *Eco*RI site present in the *env* gene. The four constructions (63-2, 63-7, 63-6, and 63-3) represent the four different possible arrangements of the *src* gene within the amphotropic viral genome. The four clones vary in the position of the *src* gene with respect to that of the LTR and in the polarity of the *src* gene with respect to that of the LTR. pBR322 sequences are not shown here, but the indicated constructions have been inserted at the *Eco*RI site in pBR322. The *Hin*dIII site in pBR322 is located 29 bases from the *Eco*RI site at the left-hand end of each construction, as shown in this figure. The *Eco*RI insert of pSRC 2 is 3.0 kb, and the *Eco*RI insert of AMT8 is 8.2 kb.

ments of the *src* gene within the amphotropic viral DNA were isolated. These clones vary in the position of the *src* gene with respect to that of the LTR (clone 63-2 versus clone 63-13) and in the polarity of the *src* gene with respect to that of the LTR (clone 63-2 versus clone 63-7). The assignment of the position and direction of the *src* gene was determined by digesting the DNAs with *Eco*RI or *Hin*dIII, transferring the DNAs to nitrocellulose filters, and hybridizing the blots with either *src*-specific probes or a probe specific for the 5′ end of the *env* gene (data not shown).

Recovery of a transforming virus. The four recombinant clones shown in Fig. 1 were cotransfected into NIH 3T3 cells with *Eco*RI-cut AMT8 DNA, which served as a source of helper virus for the rescue of the recombinant virus. In addition, unligated *Eco*RI-cut AMT8 DNA and *Eco*RI-cut pSRC2 DNA were cotransfected into NIH 3T3 cells. No changes in cellular morphology were observed in the recipient cells. Three weeks after transfection, polymerase-positive virus particles were detected in the supernatant fluids of the transfected cells, indicating that the helper virus was replicating in these cells. Supernatant fluids from these cultures were then screened for the presence of infectious transforming virus by infecting fresh NIH 3T3 cells. Foci of transformed cells were observed after 4 to 7 days in cultures receiving supernatant fluids from cells transfected with clone 63-2 plus *Eco*RI-cut AMT8, clone 63-13 plus *Eco*RI-cut AMT8, and unligated, *Eco*RI-cut pSRC2 plus *Eco*RI-cut AMT8 DNAs. No transforming virus was recovered from the supernatant fluids of cells which received *Eco*RI-cut AMT8 DNA plus clones 63-6 or 63-7. The *src* genes of clones 63-2 and 63-13 are in the same polarity (5′ to 3′) as the LTR of the amphotropic viral DNA, whereas the polarity of the *src* gene of clones 63-6 and 63-7 is opposite to that of the amphotropic virus. The recovered virus was capable of causing morphological transformation of mouse cells, mink cells, and the human epithelial cell line A431 (data not shown). The presence of $pp60^{src}$ in transformed mouse cells and A431 cells was confirmed by immunoprecipitation of radiolabeled cell extracts (data not shown).

Since the constructed virus should yield a replication-defective transforming virus, we tried to isolate clones of nonproductively transformed NIH 3T3 cells. NIH 3T3 cells were infected with supernatant fluids from the trans-

fected cells and cloned in soft agar. Colonies of transformed cells were picked after 10 to 14 days. The supernatant fluids from such clones were screened by the polymerase assay for the presence of virus particles and by infection of fresh NIH 3T3 cells for the production of infectious transforming virus. A number of clones were isolated which did not produce infectious transforming virus. Some of these clones, such as 2-1 and 18-31, produced noninfectious, polymerase-positive virus particles (Table 1), indicating that the virus present in these cells contains both functional *gag* and *pol* genes, analogous to cells transformed by the Bryan strain of RSV (18, 26, 48). The Bryan strain of RSV contains functional *gag* and *pol* genes but not a functional *env* gene, resulting in the production of a noninfectious, polymerase-positive virus particle. In this paper, we refer to both types of clones, those which produce noninfectious, polymerase-positive particles and those that do not, as nonproducer cells, since they do not produce biologically active transforming virus. Clone numbers with a prefix of 2 were derived from virus produced by transfection of cells with DNA clone 63-2 plus *Eco*RI-cut AMT8 DNA, whereas those with a prefix of 18 were derived from virus produced by transfection of cells with unligated *Eco*RI-cut pSRC2 and *Eco*RI-cut AMT8 DNAs. Infectious transforming virus could be recovered by superinfecting these cells with a variety of MuLVs, including Friend MuLV (F-MuLV), Moloney MuLV (Mo-MuLV), Friend MCF, and the amphotropic virus 4070A.

Analysis of proteins present in nonproducer cells. To determine what viral gene products were encoded by the isolated *src* recombinant viruses, nonproducer clones were labeled with [^{35}S]methionine and immunoprecipitated with a variety of specific antisera. Two typical nonproducer clones, clones 2-1 and 18-22, are shown in Fig. 2. The only virus-related proteins found in all clones were Pr65^{gag}, the precursor to the *gag* proteins (Fig. 2, lanes B and G); Pr180$^{gag\text{-}pol}$, the precursor to the enzyme reverse transcriptase (Fig. 2, lanes B and G); and pp60^{src} (Fig. 2, lanes E and J). The presence of Pr180$^{gag\text{-}pol}$, is also confirmed by the fact that these two clones both produce noninfectious, polymerase-positive virus particles (see above and Table 1).

The pp60^{src} protein of the recombinant viruses comigrated with authentic pp60^{src} from NIH 3T3 cells transformed by the Schmidt-Ruppin D strain of RSV (data not shown). No MuLV gp70 was observed in any of the nonproducer cell lines (Fig. 2, lanes C and H). A protein of approximately 32,000 daltons is shown in Fig. 2, lanes C and H; however, this protein was not observed in all nonproducer clones, and its appearance did not relate to any detectable biological properties of the viruses produced by

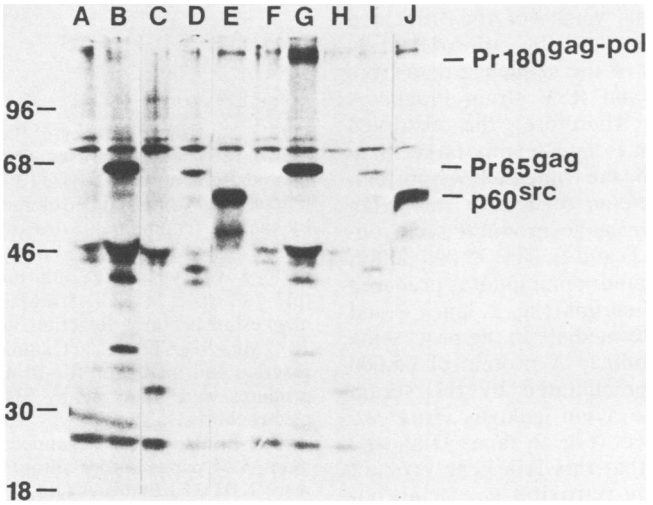

FIG. 2. Analysis of proteins present in nonproducer cell clones. The autoradiogram shows two typical nonproducer cell clones, 2-1 (lanes A through E) and 18-22 (lanes F through J). Immunoprecipitation was carried out with nonimmune serum (lanes A and F), anti-MuLV p15 serum (lanes B and G), anti-Rauscher MuLV gp 70 (lanes C and H), anti-avian leukosis virus serum which detects the *gag* and *env* gene products of avian retroviruses (lanes D and I), and tumor-bearing rabbit serum which precipitates pp60^{src} (lanes E and J). The positions of marker proteins (molecular weight $\times 10^3$) are shown on the left, and the positions of viral proteins of interest are shown on the right.

TABLE 1. Identification of nonproducer clones which release noninfectious, polymerase-positive virus particles

Clone type and no.	Focus assay[a]	cpm in polymerase assay[b]
Nonproducer		
2-1	−	20,220
2-3	−	3,362
2-25	−	1,465
18-7	−	2,280
18-21	−	1,255
18-22	−	37,910
18-31	−	45,085
Producer (Ampho 4070A infected)		
2-9	+	123,450
18-2	+	49,210
18-3	+	107,780
NIH 3T3 cells		
Mo-MuLV infected	−	225,665
Uninfected	−	1,225

[a] NIH 3T3 cells were infected with 1.0 ml of tissue culture fluid and observed for morphological transformation. Infection included 4 mg of polybrene per ml to enhance the infectivity of the virus.

[b] Counts per minute per 10 ml of tissue culture supernatant fluid incorporated in the exogenous template reverse transcriptase assay, using poly r(A · U) (52). The producer clones were infected with the amphotropic virus 4070A in addition to the *src* recombinant viral genome.

clones observed to contain this protein. The theoretical molecular weight of the truncated *env* gene product would be approximately 21,000, on the basis of the sequence data from Moloney MCF (9) and RSV strain Prague A (71a) (see below). Therefore, the observed 32,000-dalton protein is significantly larger than would be expected for the truncated *env* protein. In addition, no proteins related to the avian retrovirus *gag* or *env* gene products were observed (Fig. 2, lanes D and I). The 35,000-dalton protein seen in the immunoprecipitates prepared with anti-MuLV p15 serum (Fig. 2, lanes B and G) is probably an intermediate in the processing of the *gag* gene product. A protein of 65,000 daltons is immunoprecipitated by the serum which recognizes the avian leukosis virus *gag* and *env* gene products (Fig. 2, lanes D and I). We do not believe that this is a gene product encoded by the avian retroviral *gag* gene, because we have seen it in NIH 3T3 cells which do not contain the *src* recombinant viral genome (data not shown). The only viral proteins consistently observed in all nonproducer clones were $Pr65^{gag}$, $Pr180^{gag\text{-}pol}$, and $pp60^{src}$.

Kinase activity of the *src* protein from the recombinant virus. The $pp60^{src}$ protein of RSV is active as a protein kinase in the immune complex protein kinase assay (16, 36). We sought to demonstrate that this activity of the *src* protein had not been altered by cloning the gene into the amphotropic MuLV genome. The immune complex protein kinase assay was performed on seven independently isolated clones of cells transformed by the recombinant virus. The results indicate that the levels of kinase activity present in these cells were higher than those seen in normal NIH 3T3 cells. The level of kinase activity seen in uninfected NIH 3T3 cells (Fig. 3, lane B) is indicative of the normal cellular levels of c-*src* in these cells. The kinase activity present in the cells transformed by the *src* recombinant was always higher than those observed in uninfected NIH 3T3 cells (Fig. 3, lanes C through I versus lane B). In other experiments, kinase levels seen with clone 18-17 have been equivalent to those of any other isolate (data not shown). Furthermore, the lev-

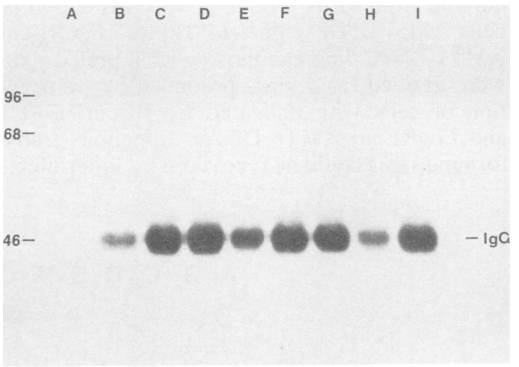

FIG. 3. Kinase activity of the $p60^{src}$ proteins from the *src* recombinant viruses. $p60^{src}$ was immunoprecipitated from normal NIH 3T3 cells and from seven independent nonproducer clones of cells transformed by the *src* recombinant viruses. Immune complexes were isolated as described in the text and were then washed with kinase reaction buffer (50 mM HEPES [pH 7.0], 0.15 M KCl, 5 mM dithiothreitol, 10 mM magnesium acetate). Reaction buffer (25 μl) containing 10^{-7} M[γ-^{32}P]ATP (3,000 Ci/mmol) was added to each reaction and incubated for 10 min at 37°C. Reaction products were analyzed by SDS-polyacrylamide gel electrophoresis. The gel was fixed, stained with Coomassie brilliant blue, destained, and dried on Whatman 3MM paper before autoradiography. Cells used were NIH 3T3 (lanes A and B), clone 2-1, (lane C), clone 2-3 (lane D), clone 2-14 (lane E), clone 2-25 (lane F), clone 18-8 (lane G), clone 18-17 (lane H), and clone 18-22 (lane I). Immunoprecipitation was carried out with nonimmune serum (lane A) and tumor-bearing rabbit serum (lanes B through I). Positions of marker proteins (molecular weight × 10^3) are shown on the left, and the positions of the heavy chain of immunoglobulin G (IgG) is shown on the right.

els of pp60^{src} in clone 18-17 were equivalent to those seen in any other nonproducer cell clone when measured by incorporation of [^{35}S]methionine into pp60^{src}. Thus, it is apparent that the pp60^{src} encoded by the recombinant murine retrovirus is still active as a protein kinase and that this activity has not been altered by the manipulations involved in cloning this sequence into a new virus vector. It is interesting to note that we did not observe autophosphorylation of pp60^{src} in the kinase assay, even when two different preparations of tumor-bearing rabbit serum were used. Autophosphorylation has been described as being an inherent property of pp60^{src} (47).

Biological activity of the *src* recombinant virus. The biological activity of the *src* recombinant virus was analyzed by injecting 6-week-old female NIH Swiss mice with either F-MuLV or Mo-MuLV pseudotypes of the *src* recombinant virus prepared by superinfecting nonproducer cells with these helper viruses. Mice were injected intramuscularly with 0.2 ml of tissue culture fluids or intravenously with 0.5 ml of tissue culture fluids. The virus titers used for injection varied from 3×10^3 to 4×10^5 focus-forming units for the intramuscular injection to 7.5×10^3 to 1×10^6 focus-forming units for the intravenous injection. Mice injected intramuscularly were observed for sarcoma formation. To date, no sarcomas have been observed at the site of injection in any of the animals receiving the transforming virus by means of intramuscular injection; over 100 animals have been examined with F-MuLV and Mo-MuLV pseudotypes of eight independent clones of the virus (clones 2-1, 2-3, 2-14, 2-25, 18-7, 18-8, 18-17, and 18-25) and the amphotropic pseudotypes of the recombinant virus generated from the original transfected cells. Clones 2-1, 2-14, 2-25, 18-8, and 18-22 produce noninfectious, polymerase-positive particles, whereas the others do not. Therefore, this difference does not play a role in sarcoma induction. The lack of sarcomas is quite surprising since, in chickens, RSV induces sarcoma formation in 100% of the animals even with injection of as little as 1 focus-forming unit of virus (25).

Subcutaneous injection of the nonproducer cells into NIH Swiss mice did result in rapid tumor formation. Injection of 10^6 cells resulted in tumor formation in 100% of the animals within 10 days. Injection of as few as 10^4 cells resulted in tumors in 20% of the animals. Thus, although injection of mice with the virus did not result in tumor formation, cells transformed in vitro were tumorigenic in vivo. Since the cells used in these studies were nonproducers, we believe that the tumors resulted from the growth of the transformed cells and not from the rescue and spread of virus from these cells, although we cannot rule out the latter possibility.

In contrast to the absence of tumors after intramuscular injection, intravenous injection of mice gave rise to splenic foci after 2 weeks and pronounced splenomegaly after 4 to 8 weeks. The spleens from mice infected with Mo-MuLV pseudotypes of three independent isolates of the *src* recombinant virus are shown in Fig. 4, along with the spleens from Mo-MuLV-infected control animals and uninfected control animals. Splenomegaly was apparent by 6 to 8 weeks. The increased spleen size could clearly be seen by 8 weeks postinfection (Fig. 4). Splenic foci were clearly observable by 2 weeks (Fig. 5). The foci present on the spleens of mice infected with the *src* recombinant virus are similar to those seen after infection of mice with either Harvey

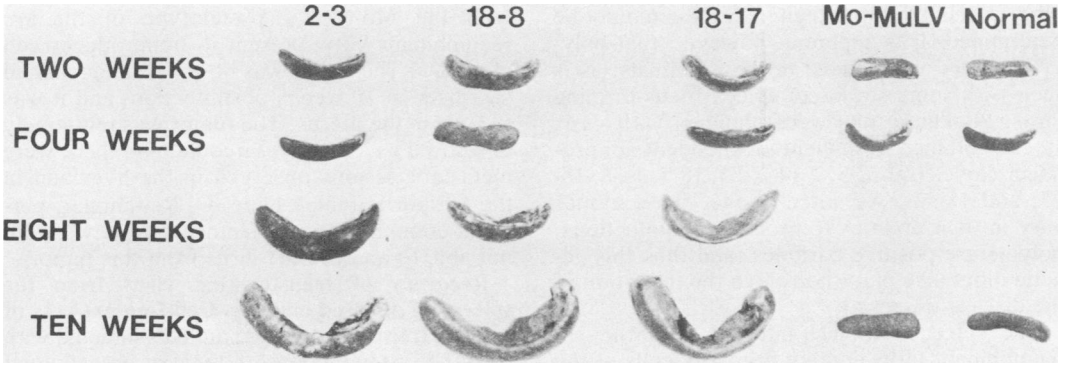

FIG. 4. Effect of the rescued *src* recombinant virus on the spleen sizes of infected mice. The nonproducer clones were superinfected with Mo-MuLV. Six-week-old NIH Swiss female mice were infected with 0.5 ml of tissue culture fluids via the tail vein. Mice were sacrificed at 2-week intervals, and the spleens were fixed in Bouins fixative. The sizes of the spleens from Mo-MuLV-infected animals and normal control animals at 6 weeks postinfection was the same as that at 4 weeks postinfection. Mice were infected with the following amounts of virus (expressed in tissue culture focus-forming units): 2-3, 1.4×10^5; 18-8, 2.4×10^5; 18-17, 8×10^3.

FIG. 5. Splenic foci present on a spleen from a mouse infected 2 weeks previously with the *src* recombinant virus rescued from nonproducer clone 2-14 with Mo-MuLV. The spleen shown is from an animal that was included in the same study as that shown in Fig. 4, although the spleens from mice infected with the virus from nonproducer clones 2-14 are not shown in Fig. 4.

or Kirsten sarcoma viruses. These foci are somewhat larger than those seen with the polycythemic strain of Friend spleen focus-forming virus, and they also have less distinct boundaries.

Table 2 shows data on mice infected with Mo-MuLV pseudotypes of eight independent clones of *src* recombinant viruses. Two animals were examined at each time point, and Table 2 shows spleen weight, hematocrit levels, and the presence of splenic foci. The degree of splenomegaly varied from isolate to isolate, perhaps owing to variation in the input titer of the virus used in each infection. By 8 weeks after infection, the hematocrit levels of some animals began to fall, and by 10 weeks after infection, anemia was observed in nearly all animals with splenomegaly. Whether the anemia was a direct effect of the virus or related to the spenomegaly could not be determined. It is apparent, however, that polycythemia is not induced in these animals, as is seen with some strains of spleen focus-forming virus. Similar results were obtained with virus stocks obtained from eight independent nonproducer clones (2-1, 2-3, 2-14, 2-25, 18-7, 18-8, 18-17, and 18-22). As noted above, these clones vary in their abilities to produce noninfectious, polymerase-positive particles, and thus this genetic difference plays no role in the induction of the disease observed.

The spleens of mice infected with the *src* recombinant virus contain primarily cells of the erythroid lineage, as determined by touch prints prepared from these spleens and by examination of cytospin preparations of cell suspensions prepared from these spleens (data not shown). No circulating erythroblasts were observed in the peripheral blood of the diseased animals, although there were large numbers of circulating reticulocytes in animals with anemia (data not shown).

Similar results were obtained with both Mo-MuLV and F-MuLV pseudotypes of the recombinant virus, although by 8 weeks, the F-MuLV control animals began to show signs of anemia and splenomegaly typical of Friend disease (40, 41). In addition, amphotropic virus 4070A pseudotypes of the recombinant viruses generated by the cells originally transfected with the DNA material were also capable of inducing this same pathological response. Amphotropic virus 4070A has not been observed to elicit any erythroproliferative disease in mice (A. Oliff, S. Ruscetti, and E. Scolnick, personal communication).

We observed one sarcoma in a mouse injected with the Mo-MuLV pseudotype of the *src* recombinant virus present in nonproducer cell clone 2-3. The tumor was observed in an animal sacrificed at 10 weeks postinfection, and it was located in the uterus. The tumor was tentatively diagnosed as a leiomyosarcoma, and there were metastatic lesions observed in the liver and in the periportal lymph node (C. Rettenmier, personal communication). Unfortunately, we were not able to analyze the virus from this tumor.

Recovery of transforming virus from the spleens of diseased animals. Cell-free extracts of spleens from two diseased animals infected with F-MuLV pseudotypes of the *src* recombinant virus were prepared and used to infect NIH 3T3 cells. Foci of transformed cells were observed after 7 days. No foci were observed in cultures receiving cell-free extracts from normal control animals. The cells were transferred to spread the virus and to increase the number of transformed

cells and labeled with [^{35}S]methionine for analysis of the viral transforming proteins present in these cells. The results (Fig. 6) indicate that only pp60^{src} was present in elevated levels in the transformed cells as compared with cells infected with extracts from control animals. No gp52, the transforming protein of Friend spleen focus-forming virus (19, 37, 51), or proteins related to MCF gp70 were observed after immunoprecipitation with an antiserum specific for MCF gp70 (Fig. 6, lanes B and F). The gp70 of the helper virus F-MuLV was not observed because anti-

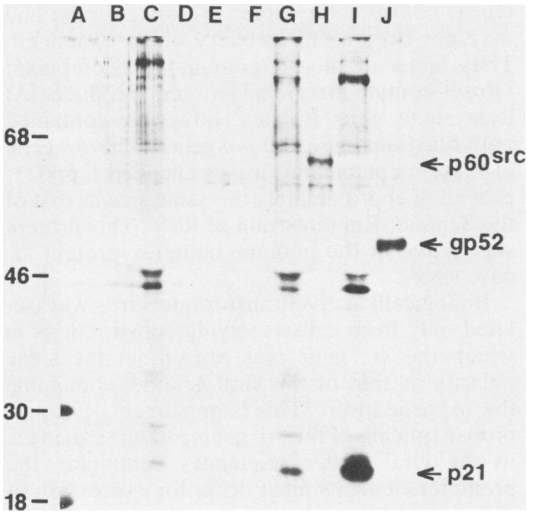

FIG. 6. Immunoprecipitation of NIH 3T3 cells infected with a cell-free extract from either a normal control animal (lanes A through D) or a mouse infected with the virus rescued from clone 2-25 with F-MuLV (lanes E through H). NIH 3T3 cells were infected with 100 μl of a cell-free 50% suspension of the spleen extract. The cells were transferred once to spread the virus and then labeled for 4 h with 200 μCi of [^{35}S]-methionine in 2 ml of methionine-free medium. Immunoprecipitates were prepared as described. Analysis of the immunoprecipitates was done on a 10% SDS-polyacrylamide gel which was processed for autoradiography after treatment with En^{3}Hance. Immunoprecipitation was carried out with nonimmune serum (lanes A and E), antiserum directed against MCF gp70 (lanes B and F), monoclonal anti-p21 259 (lanes C and G), and tumor-bearing rabbit serum, (lanes D and H). The positions of marker p21^{ras} and spleen focus-forming virus gp52 are shown in lanes I and J, respectively. Marker p21^{ras} and spleen focus-forming virus gp52 were immunoprecipitated from Harvey sarcoma virus, and spleen focus-forming virus nonproducer cells were immunoprecipitated with the monoclonal anti-p21 and with anti-MCF gp70 serum. The positions of marker proteins are indicated on the left (molecular weight × 10^3), and the positions of viral proteins of interest are indicated on the right.

TABLE 2. Effects of *src* recombinant virus on infected animals

Clone[a]	Effects at following time (wk)[b]: 2			4			6			8		
	Spleen wt	Hemat. (%)	Splenic foci	Spleen wt	Hemat. (%)	Splenic foci	Spleen wt	Hemat. (%)	Splenic foci	Spleen wt	Hemat. (%)	Splenic foci
2-3	0.19	44	+/−	0.39	46.5	−/−	1.06	41	+++/+++	1.02	31.5	+++/+++
18-8	0.33	45.5	+++/−	0.17	48	+/−	0.30	47	+++/−	1.46	35	+++/+++
18-17	0.22	45	+++/−	0.20	45.5	+/−	0.50	41.5	+++/−	1.1	34	+++/+++
Mo-MuLV	0.10	47	−/−	0.15	46	−/−	0.16	46.5	−/−	0.14	48	−/−/−
Uninfected	0.10	47	−/−	0.10	47	−/−	0.11	48.5	−/−	0.2	49	−/−/−

[a] Mo-MuLV-superinfected NP clones were used for these studies.

[b] A 0.5-ml amount of a 24-h tissue culture supernatant fluid was injected intravenously via the tail vein into 6-week-old female Swiss mice. Supernatant fluids were filtered through a 0.45-μm membrane filter (Millipore Corp.) before injection into animals. Animals were examined at the times indicated postinfection. Spleen weight (in grams) and hematocrit levels (Hemat.) are the averages of two animals at each time point. Some spleens from this experiment are shown in Fig. 4. Results for splenic foci are shown for two mice and are separated by a slash. +, 1 to 10 foci per spleen; ++, 10 to 200 foci per spleen; +++, too many to count, confluent.

bodies which recognize ecotropic MuLV gp70 had been removed by absorbing the antiserum.

Harvey and Kirsten sarcoma viruses, which encode p21^{ras} (54), are able to induce erythroleukemia in mice (53). Therefore, the level of p21^{ras} in cells transformed by the recovered virus was examined to exclude the possibility that a virus encoding p21 had been rescued. p21^{ras} was present in both transformed cells and normal control cells (Fig. 6, lanes C and G). Although there appears to be a slight increase in the amount of p21^{ras} in lane G versus lane C, we do not believe that this increase is significant enough to indicate that a p21-encoding virus is responsible for the observed disease. Furthermore, this difference was not observed in other experiments in which we have recovered virus from the tissues of infected animals. Further experiments are in progress to eliminate the possibility that a *ras*-containing virus is involved in the observed disease. Only the level of pp60^{src} was vastly increased in transformed cells as compared with the control cells (Fig. 6), lane D versus lane H). These results indicate that a transforming virus encoding pp60^{src} can be recovered from the spleens of diseased animals. Viruses encoding p21 or MCF-related glycoproteins, including gp52 of spleen focus-forming virus, were not recovered from these spleens. The observed erythroleukemia thus does not involve the *ras* gene of either Harvey or Kirsten sarcoma viruses, two viruses which can induce erythroleukemia in mice (53). In addition, our inability to observe either gp52 of spleen focus-forming virus or MCF-related gp70 rules out the involvement of these types of viruses in the disease studied here. Spleen focus-forming virus is responsible for the rapid erythroleukemia induced by the Friend virus complex (65). MCF viruses may play a role in the induction of anemia by F-MuLV and are found in erythroid cell lines established from such animals (41).

We have not been able to directly immunoprecipitate pp60^{src} from [^{35}S]methionine-labeled spleen cells from diseased animals. We assume that the levels of pp60^{src} are not high enough or the background of labeled cellular proteins is too high to permit its observation. Unfortunately, the anti-pp60^{src} serum available to us apparently does not recognize denatured pp60^{src}, and therefore, we are not able to detect pp60^{src} by "Western transfer" of proteins to nitrocellulose filters and binding of antibody to these filters.

DISCUSSION

We report here the isolation of a murine transforming virus containing the *src* gene of SRA-RSV. The *src* gene was inserted into the *env* region of the replication-competent virus amphotropic MuLV 4070A, thus generating a replication-defective transforming virus. The *Eco*RI site present in the *env* region of MCF and amphotropic MuLVs is highly conserved (13). A comparison of the reading frames and sequences surrounding this *Eco*RI site in the *env* gene of Moloney MCF (9) and the *env* gene of RSV Prague C (71a) indicates that this fusion results in a 1+ change in the reading frame of the RSV *env* gene, resulting in a termination codon 12 bases after the *Eco*RI site. Therefore, the fusion Ampho MuLV *env*-RSV *env* gene product contains 191 amino acids and has a theoretical molecular weight of 21,000 daltons. The only protein observed with the anti-MuLV gp70 protein is considerably larger (32,000 daltons) and may not be present in every clone examined. Thus, none of the *src*-containing recombinant viruses contain functional *env* gene products. At least some virus isolates, however, contained both functional *gag* and *pol* genes. The *src* gene of these recombinant viruses encoded a pp60^{src} protein of approximately the same size as that of the Schmidt-Ruppin strain of RSV. This protein was active in the immune complex protein kinase assay.

Biologically active transforming virus was isolated only from cells receiving constructions in which the *src* gene was present in the same polarity as that of the viral genome containing the *src* gene insert. This is presumably because proper splicing of the *src* gene coding sequences to the viral leader sequences containing the promoter elements must occur for expression of the *src* gene.

Although injection of nonproducer cells into adult mice resulted in tumor formation, intramuscular injection of cell-free preparations of the *src* recombinant virus did not result in sarcoma formation. Instead, after intravenous injection of the virus, we observed the induction of an erythroproliferative disease characterized by splenic foci, splenomegaly, and, late after injection, anemia. We did not observe primitive erythroid cells in the peripheral blood of these animals, although anemic animals contained large numbers of circulating reticulocytes. We observed one apparent intrauterine sarcoma in an animal intravenously injected 10 weeks earlier with a Mo-MuLV pseudotype of one of the *src* recombinant viruses, indicating that, under the appropriate conditions, a higher incidence of sarcomas may be observed. An infectious transforming virus could be recovered from cell-free homogenates prepared from the spleens of diseased animals. Immunoprecipitation of labeled cell extracts prepared from cells transformed by these spleen extracts revealed that the levels of pp60^{src} were vastly increased in these cells. We observed some increase in the levels of p21^{ras} in

these cells when a broadly cross-reactive monoclonal antibody was used, although this increase does not seem to be reproducible in other preparations of recovered viruses. Also, we did not observe MCF-related glycoproteins present in cells transformed by virus recovered from spleens of diseased animals. Thus, our data indicate that a murine retrovirus containing the *src* gene of the avian retrovirus RSV can be isolated and that, under the conditions used in this study, it is capable of inducing an erythroproliferative disease.

The interaction of RSV with mammalian cells in vivo and in vitro has been extensively studied (1, 56, 57, 61, 73, 74). In most cases, sarcomas can be induced either by transplantation of chicken sarcoma material or, in a few studies, with cell-free preparations from tumors. The efficiency of tumor induction varies with the RSV strain used, the type and age of the animals, and route of inoculation. The recovery of infectious RSV from mammalian tumors or cells transformed in vitro is rare (5, 33, 58, 62). It is possible, however, to recover infectious RSV by cocultivation of mammalian cells with chicken embryo fibroblasts (56, 59, 63). Inoculation of newborn mice with minced tumor tissue gives rise to tumors in approximately 25% of the animals.(1, 4). Ahlstrom (1) noted that no tumors were observed in mice older than 10 days at the time of injection. Jonsson (30) observed sarcoma induction after injection of mice with cell-free preparations from chicken sarcomas. These tumors could be maintained by transplantation to other mice, and sarcomas could be induced in chickens with minced tumor tissue. Transfer of the sarcoma to either other mice or chickens could not be performed with cell-free extracts from the mouse sarcoma, indicating that there was no infectious transforming virus produced by these cells.

Our results differ from those of previous studies in two ways. First, we are readily able to recover infectious transforming virus from both cells transformed in vitro and tissues from diseased animals. Second, the disease spectrum we observed varies significantly. We primarily observed an erythroproliferative disease characterized by splenomegaly and anemia. We are studying the response of newborn mice to the *src* recombinant virus and of other newborn animals known to be sensitive to sarcoma induction by RSV or RSV tumor cells. Such animals include hamsters, rabbits, guinea pigs, and rats (1, 57, 74). Preliminary results indicate that the *src* recombinant virus induces sarcomas and splenomegaly in newborn mice.

The reasons behind the different pathological picture observed in this study as compared with that in previous studies is not immediately obvious. The sarcomas observed in previous studies represent the clonal outgrowth of nonproductively transformed mammalian cells which do not produce infectious virus. In our study, the *src* recombinant virus is able to replicate and spread through the host animals; thus, the response observed is polyclonal in nature. Our results, therefore, may reflect what target cell is easiest for the *src* gene to transform in mice. This approach differs significantly from those of earlier studies which asked what cells nonreplicating RSV could transform to yield a tumor.

The results described here with the *src* recombinant virus may be similar to that described for the myeloproliferative sarcoma virus which induces erythroid and myeloid proliferation, formation of splenic foci, splenic foci, and splenomegaly in adult mice (29, 32, 35, 42), although it causes sarcomas in newborn mice (14, 29). It is interesting to note that the myeloproliferative sarcoma virus was derived from Moloney sarcoma virus by forced passage in adult BALB/c mice and that it apparently contains all of the *mos* gene sequence and no additional cellular sequences that are not present in Moloney sarcoma virus (46). It is tempting to speculate that the difference in the pathological picture produced by these two closely related viruses, Moloney sarcoma virus and myeloproliferative sarcoma virus, might be due to differences in the LTRs of these two viruses.

The results of this study suggest that retroviral *onc* genes may not be as specific as has often been thought. In this respect, it is interesting to note that, in several cases, single retroviral *onc* genes transform cells in several unrelated tissue types. For example, the *ras* gene of Harvey, Kirsten, and Balb sarcoma viruses can induce erythroleukemia in mice (53), despite the fact that all three viruses were isolated from sarcomas in mice or rats (28, 31, 44). In vitro, these viruses transform mouse fibroblasts, erythroid cells (27), and early pre-B lymphoid progenitor cells (45). Similar findings have been noted for Abelson murine leukemia virus, which transforms lymphoid cells (49), mouse fibroblasts (53a), and erythroid precursors present in fetal liver cells (70).

Further experiments are under way to examine the effects of the *src* recombinant viruses in other mammalian species. We also hope to establish pseudotypes of the *src* recombinant virus which are infectious for chicken cells to determine whether the *src* recombinant viruses are still able to induce sarcomas in chickens. We are also examining the effects of the *src* recombinant viruses upon hematopoetic cell populations by means of in vitro assays to determine what types of cells these viruses are capable of transforming. Attempts to isolate temperature-sensitive

mutants of the *src* recombinant virus are in progress. Isolation of such mutants should help to clearly demonstrate the role of the *src* gene in the induction of the erythroid disease.

ACKNOWLEDGMENTS

We thank J. M. Bishop for the gift of the pSRC 2 clone and H. Opperman, H. Hanafusa, S. Ruscetti, and M. Furth for gifts of antibodies used in these studies. The excellent technical assistance of Bob Lowe and Bill Vass is gratefully acknowledged.

S.M.A. was a postdoctoral fellow of the Damon Runyon-Walter Winchell Cancer Fund.

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