Recombinant BALB and Harvey Sarcoma Viruses with Normal Proto-*ras*-Coding Regions Transform Embryo Cells in Culture and Cause Tumors in Mice

KLAUS CICHUTEK AND PETER H. DUESBERG*

Department of Molecular Biology, University of California, Berkeley, California 94720

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The *ras* genes of BALB and Harvey sarcoma viruses contain point mutations in codon 12 or codons 12 and 59, relative to proto-*ras* from normal animal and human cells. By in vitro recombination between cloned rat proto-*ras* and cloned BALB and Harvey sarcoma proviruses, we constructed recombinant proviruses with normal proto-*ras*-coding regions. These recombinant proviruses transformed mouse 3T3 cells upon transfection. However, when the transforming efficiencies of proviral DNAs were compared after transfection with helper provirus, recombinant proviruses were 2 to 30 times less efficient than the corresponding wild-type proviruses. Recombinant sarcoma viruses isolated from cells transformed by cloned proviral DNA contained the expected normal *ras*-coding region. They transformed rat embryo cells and induced erythroblastosis and sarcomas in newborn mice as efficiently as wild-type viruses did. We conclude that conversion of normal proto-*ras* genes to viral *ras* genes depends on truncation of normal proto-*ras* regulatory elements and substitution by retroviral (long terminal repeat) promoters and that the transforming function of long terminal repeat-*ras* genes is enhanced by point mutations.

Murine sarcoma viruses with transforming *ras* genes cause erythroblastosis or fibrosarcomas in rats and mice and transform human and a variety of animal cells in vitro (3, 5). They are sufficient for initiation and maintenance of transformation of cells in culture and of tumors in animals (16, 21, 35). Viral *ras* genes encode a single transforming protein, p21^{ras}, that is colinear with a cellular *ras* protein (6-8, 13-15, 17, 24, 32) or a hybrid protein composed of retroviral and *ras* elements, e.g., p29^{Δgag-ras} (27). Thus, the coding region of viral *ras* genes is either entirely (p21) or partially (p29) transduced from normal cellular genes termed proto-*ras* (7, 13, 17), whereas the promoters are derived from retroviruses (11).

There are two competing hypotheses to explain the conversion of a normal proto-*ras* gene to a cancer gene like the viral *ras* genes.

(i) Proto-*ras* genes from certain tumors morphologically transform mouse NIH 3T3 cells upon transfection due to a point mutation in codons 12, 13, 59, or 61 (3, 6, 29, 40). Since point mutations in codons 12 and 59 are also found in viral *ras* genes, it has been proposed that both viral *ras* genes (4, 19, 27, 28, 39, 42) and cellular proto-*ras* genes (6, 29, 40) derive their transforming function from specific point mutations relative to normal proto-*ras*. This hypothesis, however, cannot be reconciled with the existence of such point mutations in benign clonal hyperplasias of animals (2, 30) and with the discrepancy between the much higher probabilities of occurrence of point mutations than of cancers in animals (16). It also fails to explain why NIH 3T3 cell-transforming proto-*ras* genes with point mutations do not transform primary cells (23, 26, 31, 33), whereas viral *ras* genes do (11).

(ii) A competing hypothesis proposes that viral and possibly cellular *ras* genes acquire transforming function by truncation of the cellular promoter and substitution for a viral or an equivalent heterologous promoter (11, 16). This

hypothesis is based on experiments which show transforming function of recombinant Harvey sarcoma viruses or recombinant *ras* genes containing normal proto-*ras*-coding regions artificially linked to a retroviral promoter (long terminal repeat [LTR]) (8, 9, 11). Since only *ras* genes under the control of heterologous retroviral promoters transform diploid cells in vitro, it has been proposed that truncation and promoter substitution, which involve a marked genetic alteration of proto-*ras*, are necessary to achieve the high level of p21 expression required for transformation of primary cells. This hypothesis explains why native proto-*ras* genes with point mutations do not transform normal diploid cells and why viral *ras* genes transform diploid cells with or without point mutations (11).

We tested the hypothesis that conversion of proto-*ras* to a primary cell-transforming gene depends on the substitution of the native proto-*ras* promoter by a heterologous promoter. We demonstrated transforming function in diploid cells and oncogenicity in mice of recombinant BALB and Harvey sarcoma viruses (BalbSV and HaSV, respectively) expressing normal p21^{ras}. Furthermore, we have shown that recombinant viruses retained normal proto-*ras* codons upon transformation of cells in vitro.

MATERIALS AND METHODS

Cloning of proviruses. A BalbSV provirus was constructed from plasmid pP-7 (1). pP-7 represents a *Pst*I insert of BalbSV provirus into plasmid pBR325. This provirus contains a complete 5' LTR but only a defective 3' LTR (1). To restore the 3' LTR, most of the *Pst*I insert of pP-7 was deleted from a *Pvu*II site near the 5' end to the *Pvu*II site in pBR325. Subsequently, the *Pst*I BalbSV insert of pP-7 was reinserted to generate pB (Fig. 1).

Provirus pB12 with a normal proto-*ras*-derived codon 12 (Fig. 1) was constructed by exchange of a 95-base-pair (bp) *Sac*I (nucleotide [nt] · 112)-*Pvu*II(207) fragment of pB (Fig. 1) against the corresponding fragment of pcras1 (13), a rat Harvey proto-*ras* 1 DNA clone (Fig. 1). For this purpose, pB

* Corresponding author.

FIG. 1. Structures of wild-type and recombinant BalbSVs and rat Harvey proto-*ras* 1. Locations of restriction enzyme sites (in base pairs) based on structural and sequence analyses of BalbSV (1, 28) and rat Harvey proto-*ras* 1 (11, 32) are indicated. pB is wild-type BalbSV. pB12 is a recombinant with a normal proto-*ras*-specific codon 12, and pBpras1 is a recombinant with a normal proto-*ras*-derived coding region. Symbols: donor and acceptor are splice signals; term represents a stop codon; poly(A) is a poly(A) addition site; restriction sites: B, *Bam*HI; F, *Fsp*I; H, *Hind*III; Hi, *Hinc*II; Pv, *Pvu*I; P, *Pvu*II; Ps, *Pst*I; S, *Sac*I; Sp, *Spe*I; and X, *Xba*I. The 12 represents the virus-specific *ras* codon of BalbSV. Boxes represent transcribed viral or proto-*ras* sequences: stippled boxes are AKR retroviral sequences (1, 5, 28), open boxes are viral *ras*, and solid boxes are normal rat Harvey proto-*ras* coding region (exons I to V are indicated). For construction of proviruses, see Materials and Methods.

was first cut at the unique *Sac*I site 5' of *ras* and the unique *Xba*I site 3' of *ras*. After removal of the insert, the vector was ligated to the 95-bp *Sac*I(131)-*Pvu*II(226) fragment of pcras1 and a *Pvu*II-*Xba*I fragment derived from pB. The inserted pcras1-derived fragment contains the translation start codon of p21^{Ha-ras} and the normal proto-*ras* codon 12 (13).

To generate a BalbSV with a complete proviral proto-*ras* coding region, the *Hind*III(156)-*Xba*I fragment of pB that contains the viral *ras* coding region was exchanged against a corresponding *Hind*III(175)-*Xba*I fragment of pcras1 (13). For this purpose, a *Sac*I(112)-*Hind*III(156) fragment of pB and the *Hind*III-*Xba*I fragment of pcras1 were ligated with the *Xba*I-*Sac*I(112) fragment of pB which included the complete pBR325 vector. The resulting provirus, pBpras1, contained proto-*ras* exons II to V, including the respective introns (Fig. 1).

Cells and viruses. Mouse NIH 3T3 cells were grown from stocks kindly provided by E. Scolnick. DME medium (Gibco Laboratories, Grand Island, N.Y.) containing 5 to 10% calf serum (Gibco) was supplemented with penicillin, streptomycin, and mycostatin. Primary rat embryo fibroblasts (Whittaker Bioproducts, Walbersville, Md.) were grown in DME containing 10% fetal calf serum (Gibco).

DNA transfection by calcium phosphate precipitation was done as described before (11). Typically, 1 μ g of sarcoma proviral DNA and 0.5 to 1 μ g of Moloney murine leukemia virus (MoMLV) proviral DNA (pZap) (37) were ethanol precipitated in the presence of 10 μ g of sheared herring sperm DNA as a carrier. DNA was redissolved in 500 μ l of 2 $\times$ HBS (2 $\times$ HBS is 280 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.0]); 500 μ l of 0.25 M CaCl₂ was added to form precipitates, which were then added to about 2.5×10^6 cells in 10 ml of growth medium supplemented with 10% fetal calf serum. After 12 to 18 h, the medium was changed, and cells were allowed to grow to confluency, after which they were split 1:6. The time required for the appearance of the first transformed cells is referred to below as the latency period. Foci were scored 3 to 30 days later. Culture medium containing viruses was frozen and thawed and then passed through 0.45- μ m-pore-size Millipore filters before they were used to infect other cells. Rat embryo fibroblast cells were seeded at 5×10^5 cells per 100-mm dish and infected with 1 ml of filtered medium in the presence of 5 μ g of Polybrene per ml.

Sequence analysis of proviral DNA. Foci of cells transformed by transfection with the Harvey provirus pA1259 in the presence of helper Moloney provirus (pZap) (11) were grown into mass cultures. Virus from clonal cultures was harvested and used to infect mouse NIH 3T3 cells. Proviral DNA from 5×10^5 NIH 3T3 cells infected with 15 ml of undiluted virus stock was prepared about 30 h after infection by standard procedures (20). After digestion with restriction enzymes *Sac*I and *Xba*I, fragments spanning 0.8 to 1.4 kilobases (kb), which would include the 1.2-kb *Sac*I-*Xba*I Harvey proviral DNA fragment (Fig. 1), were isolated by preparative agarose gel electrophoresis. The fragments were then ligated into lambda phage vector λ Zap (Stratagene, La Jolla, Calif.), previously digested with *Sac*I and *Xba*I. Subgenomic libraries were screened by hybridization (25) with a ³²P-labeled 0.7-kb *Hind*III-*Pst*I fragment of Harvey provirus pA (11). *Hind*III-*Pst*I fragments (15) from positive λ clones were analyzed by Sequenase sequencing (US Biochemicals, Cleveland, Ohio) in vector M13mp18.

Inoculation of mice. Virus-free BALB/c mice (Simonsen, Gilroy, Calif.) were injected intraperitoneally or in the back with 50 to 100 μ l of virus-containing growth medium solution 2 to 3 days after birth (see figure legends for titers). Litters injected with identical viruses were kept separate. Any moribund animal or animal with a tumor on its back was sacrificed, and tumors in the spleen or sarcomas on the back were diagnosed after necropsy.

Analysis of viral RNAs and protein. Viruses were pelleted from 250 ml of cell medium by centrifugation at 19,000 rpm for 2 h in a Beckman type 19 rotor. Precipitated virus was redissolved in 1 ml of STE buffer (25), and loaded onto 3-ml 15 to 30% sucrose gradients, and centrifuged in a Beckman SW55 rotor for 30 min at 45,000 rpm. The virus band (1.16-g/ml density) was diluted with TE (25), and viral RNA was isolated by phenolization in the presence of 1% 2-mercaptoethanol and 1% sodium dodecyl sulfate (SDS) and subsequent ethanol precipitation. Isolation of cytoplasmic RNA from transformed NIH 3T3 cells and electrophoretic analysis were performed according to standard procedures (25).

For protein analysis, about 10^5 virus-transformed NIH 3T3 cells were incubated at 37°C for 18 h in methionine-free medium containing 200 μ Ci of [³⁵S]methionine (1,134 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml

and 3% dialyzed calf serum. Cell lysates were prepared as described before (35) and immunoprecipitated with rat monoclonal antibody Y13-238 specific for p21^{Ha-ras} or p21^{N-ras} by published procedures (24, 35).

RESULTS

Recombinant sarcoma viruses with proto-*ras*-coding regions. To determine its possible role in the transforming function of sarcoma viruses, we exchanged the virus-specific *ras* codon 12 of BalbSV for the equivalent codon of proto-*ras* by in vitro recombination with normal rat proto-*ras*. Prior to mutagenesis, the defective 3' LTR of the proviral clone pP-7 (1) was repaired by adding the missing LTR elements from the 5' LTR to construct pB (Fig. 1) (see Materials and Methods). The BalbSV-specific Lys of *ras* codon 12 was then replaced by the proto-*ras*-specific Gly by exchanging a 95-bp *Sac*I(112)-*Pvu*II(207) *ras* region of pB against its rat proto-*ras* 1 equivalent *Sac*I(131)-*Pvu*II(226) fragment (Fig. 1). This recombinant was termed pB12 (Fig. 1). Another recombinant, pBpras1, was constructed in which the complete *ras*-coding region from a *Hind*III(156) site in the *ras* coding region to an *Xba*I site downstream of *ras* was exchanged for a 2.2-kb proto-*ras* equivalent (Fig. 1).

To verify that the newly constructed wild-type and recombinant proviral clones were infectious proviruses and generated viruses that replicated like wild-type BalbSV (rather than behaving like nonreplicating *ras* DNAs upon transfection), intra- and extracellular viral RNAs of cells transfected with these constructs were analyzed. As can be seen in Fig. 2, pB and pB12 each generated the expected 6.6-kb viral RNA (1), which is larger than the 5.7-kb RNAs of wild-type HaSV (pA) and the HaSV without point mutations (pA1259) (11). The recombinant pBpras1 with a proto-*ras*-coding region generated a 6.5-kb viral RNA (Fig. 2). This was expected, because a small part of the noncoding region downstream of *ras* was deleted from wild-type provirus during the construction of this recombinant (Fig. 1). In contrast to HaSV, BalbSV has been proposed to express p21^{ras} protein via a subgenomic mRNA (28; S. A. Aaronson, personal communication) (Fig. 1). Indeed, the intracellular RNA of BalbSV-transformed cells contained a 2.9-kb (pB12) and a 2.6-kb (pBpras1) subgenomic *ras* mRNA (Fig. 2). These RNAs were not found in cells transformed by HaSV (pA or pA1259). The sizes of the subgenomic RNAs are in agreement with the assumption that the splice donor near the 5' end of the viral genome and the *env* splice acceptor, which immediately precedes the *ras* coding region of BalbSV (28), are used to generate the subgenomic 2.9-kb or 2.6-kb *ras* mRNA (Fig. 1). The molar ratio of the genomic and subgenomic BalbSV *ras* RNAs was about 1:1. It follows that our recombinant BalbSV proviruses were infectious and expressed *ras* mRNAs like those of wild-type BalbSV.

Viruses containing the coding sequence of normal proto-*ras* transform primary rat cells like wild-type sarcoma viruses. The transforming function of BalbSV proviruses, with or without point mutations compared with proto-*ras*, was first tested in NIH 3T3 cells. For this purpose, cloned proviral DNAs were transfected together with proviral MoMLV helper proviral DNA by calcium phosphate precipitation. Transforming efficiencies were compared as foci of transformed cells per microgram of proviral DNA. The results of several transfection experiments are compiled in Table 1. Proviruses pB and pB12 showed almost identical transformation efficiencies. Foci of transformed cells were visible about 7 to 10 days after transfection. The transforming

FIG. 2. Comparison of *ras*-specific extra- and intracellular RNAs from BalbSVs and HaSVs. From 1 to 2 μ g of viral RNA, 10 to 15 μ g of total cytoplasmic RNA, or 2 μ g of twice poly(A)-selected cytoplasmic RNAs were prepared for electrophoretic analysis. The following viruses were used: MoMLV, wild-type HaSV (pA), recombinant HaSV with normal proto-*ras*-specific *ras* codons 12 and 59 (pA1259), recombinant HaSV with a proto-*ras*-derived coding region (pApras1X), wild-type BalbSV (pB), recombinant BalbSV with normal proto-*ras*-specific *ras* codon 12 (pB12), and recombinant BalbSV with a normal proto-*ras*-derived coding region (pBpras1). The origin of viruses or proviral clones shown in parentheses is described in the text and in reference 11. The RNAs were subjected to electrophoresis in buffered 1% agarose gels containing formaldehyde (25). RNA was then blotted onto nitrocellulose (Gene-Screen; New England Nuclear), and *ras*-containing species were identified by autoradiography after hybridization with the 0.7-kb, *ras*-specific, ³²P-labeled *Hind*III-*Pst* fragment of Harvey provirus pA (11).

efficiency of pBpras1 was 3 to 10% of that of wild-type pB, and focus formation occurred 12 to 30 days after transfection.

The transformation efficiencies and kinetics of wild-type and recombinant BalbSV viruses were then compared with those of analogous mutants of HaSV described previously (11). Wild-type HaSV (pA) and the HaSV recombinant pA1259 (11), which is analogous to pB12 in that it contains a *ras* gene without virus-specific point mutations, induced foci

TABLE 1. Transformation efficiency of wild-type and mutant BalbSVs and HaSVs in NIH 3T3 cells^a

Virus	Transformation efficiency (FFU/ μ g of proviral DNA) ^b	Latency (days)
pB	300	7
pB12	200	10
pBpras1	10–30	12–30
pA	1,000	5
pA1259	400	7
pApras1X	50–100	12–30

^a Proviruses were introduced into mouse NIH 3T3 cells by transfection of 1 μ g of proviral DNA with 1 μ g of MoMLV helper provirus (pZap). Foci of transformed cells appeared after a latency period of 5 to 30 days. Values are averages of at least two experiments. The following proviruses were tested: wild-type BalbSV (pB), recombinant BalbSV with a normal proto-*ras*-derived codon 12 (pB12) and a normal proto-*ras*-coding region (pBpras1), wild-type HaSV (pA), and recombinant HaSV with normal proto-*ras*-derived codons 12 and 59 (pA1259) and with a normal proto-*ras*-coding region (pApras1X). See text and reference 11 for construction of the proviruses.

^b FFU, Focus-forming units.

5 to 7 days after transfection (Table 1). Focus formation by the recombinant HaSV pApras1X, which is analogous to pBpras1 in that it contains a complete proto-*ras*-coding region (11), occurred 12 to 30 days after transfection. Transforming efficiency per microgram of proviral DNA of pA1259 was 20 to 40% and that of pApras1X was 10% of that of wild-type HaSV. Thus, wild-type and recombinant BalbSV proviruses behaved like the corresponding HaSV proviruses in terms of transformation efficiencies and kinetics. The most notable distinction of the recombinant BalbSV and HaSV proviruses containing proto-*ras* introns was that they were less efficient and slower in transforming NIH 3T3 cells than those carrying viral *ras*. However, the morphological phenotypes of cells transformed by wild-type and recombinant viruses were indistinguishable (11; data not shown). Moreover, virus titers in growth medium of NIH 3T3 cells transformed by recombinant or wild-type BalbSV and HaSV were very similar, ranging from 10^4 to 10^6 cell FFU/ml.

Viral *ras* genes with normal proto-*ras*-coding regions could be expected either to transform both aneuploid NIH 3T3 cells and diploid primary cells, like wild-type viral *ras* genes, or to transform only aneuploid NIH 3T3 cells, like cellular proto-*ras* genes with point mutations. To distinguish between these alternatives, virus-containing supernatants of transformed NIH 3T3 cells was seeded on rat primary cells. All recombinant BalbSV and HaSV viruses transformed primary rat cells efficiently after 5 to 7 days (data not shown). The kinetics of focus formation by all recombinant and wild-type viruses were identical. Thus, no differences could be observed in transforming efficiencies of viruses carrying *ras* genes with and without virus-specific codons when compared in NIH 3T3 or diploid rat primary cells.

Proto-*ras* codons are conserved in recombinant sarcoma viruses that transform cells in culture. To determine whether the sarcoma viruses produced by cells transformed by recombinant proviruses with normal proto-*ras*-coding regions retained proto-*ras*-specific codons, the viral genomes and p21^{ras} proteins from transformed cells were analyzed. Direct sequence analysis of viral RNAs was found to be unreliable due to high backgrounds in sequencing gels. Therefore, unintegrated proviral DNAs from cultures infected with cloned virus stocks were analyzed. Such stocks had been obtained from clonal cultures that had been prepared from single foci of NIH 3T3 cells transformed by pA1259, an HaSV provirus with normal proto-*ras*-specific codons 12 and 59 (11). The unintegrated proviral DNA of cells infected by such virus stocks was digested with *Sac*I and *Xba*I to generate a 1.2-kb *Sac*I-*Xba*I-resistant *ras* fragment of pA1259 for molecular cloning and sequence analysis. *Sac*I- and *Xba*I-resistant DNA fragments ranging in size from 0.8 to 1.4 kb were then prepared and cloned into the lambda vector λ -Zap. *ras*-containing clones were selected by hybridization with *ras* [³²P]DNA probes, and *ras* DNAs were subjected to DNA sequence analysis as described in Materials and Methods. Proviruses from two clonal stocks of pA1259 indeed retained the input proto-*ras* 2 codons 12, 13, 59, and 61 (11, 32) (data not shown). Proto-*ras* 2-specific codons 34, 44, and 53 (11, 32) were also retained. Thus, viral *ras* genes do not depend on wild-type-specific codons for transforming function.

Wild-type HaSV expresses two electrophoretically distinct forms of p21 (35). A fraction of p21, referred to as pp21, is phosphorylated at threonine-59 (5, 15) and has a slightly lower electrophoretic mobility than its unphosphorylated counterpart. Accordingly, a double band of p21 and pp21 is detected in cells transformed by wild-type HaSV (pA). Since

FIG. 3. Immunoprecipitation of p21^{ras} proteins from NIH 3T3 cells transformed by wild-type and recombinant sarcoma viruses. Electrophoretic analysis of proteins precipitated by anti-*ras* serum from mouse NIH 3T3 cells infected by the following viruses: MoMLV, wild-type HaSV (pA), recombinant BalbSV with normal proto-*ras*-specific codon 12 (pB12) and a normal proto-*ras*-coding region (pBpras1), and recombinant HaSV with normal proto-*ras*-derived codons 12 and 59 (pA1259) and a proto-*ras*-coding region (pApras1X) (see Fig. 2 and the text for the origin of viruses). Cell lysates prepared as described in Materials and Methods were immunoreacted with Ha-*ras*-specific monoclonal antibody Y13-238 and separated by SDS-polyacrylamide gel electrophoresis (4.5% stacking gel, 15% separating gel) relative to protein standards. Arrows indicate p21^{ras} and its phosphorylated form pp21 relative to [³⁵S]methionine-labeled proteins were visualized by autoradiography after gel fixing (35).

threonine at position 59 is one of the virus-specific *ras* codons, versus the alanine of normal proto-*ras* (32), it can be used as an indicator for back mutation at codon 59. In order to determine whether a proto-*ras*-specific codon 59 was retained, we analyzed the transforming p21 proteins expressed by four recombinant proviruses (pA1259, pApras1X, pB12, and pBpras1) that all contained the proto-*ras*-specific alanine at codon 59. Protein extracts from NIH 3T3 cells transformed by each virus were immunoprecipitated with a *ras*-specific monoclonal antibody, Y12-238, which is Ha-*ras*-specific (see Materials and Methods). Figure 3 shows an autoradiogram of immunoprecipitated [³⁵S]-labeled *ras* proteins after SDS-polyacrylamide gel electrophoresis. Wild-type HaSV showed the expected two p21 proteins. Cells transformed by recombinant proviruses pB12, pBpras1, pA1259, and pApras1X expressed exclusively the nonphosphorylated form of p21, in agreement with the absence of threonine at codon 59. Cellular p21^{ras} protein was undetectable under the conditions applied and was estimated to be present in at least 20-fold-lower amounts than virus-specific p21 (compare the MoMLV track to those of cells infected by the five viruses). The proteins of >30 and >21 kilodaltons (kDa) coprecipitated by our anti-*ras* serum have been observed previously (24). Their nature is unknown.

Sarcoma viruses with proto-*ras*-coding regions cause erythroblastosis and fibrosarcomas in mice. The oncogenicity of BalbSV and HaSV with proto-*ras*-coding regions was analyzed by inoculation of newborn BALB/c mice with 10^3 to 10^4 FFU (tested in NIH 3T3 cells) of the respective recombinant viruses; wild-type viruses were used as positive controls. Wild-type HaSV is known to cause either lethal erythroblastosis or sarcomas in animals (10, 19, 22, 34). As shown in Fig. 4, all seven to nine animals inoculated with wild-type BalbSV or HaSV developed lethal erythroblastosis, and one of seven to nine mice showed fibrosarcomas. Essentially the same results were obtained with the recombinant BalbSVs and HaSVs containing normal proto-*ras*-

FIG. 4. Survival curves of mouse litters inoculated with MoMLV or wild-type and recombinant sarcoma viruses. Litters of BALB/c mice (2 to 3 days old) were inoculated intraperitoneally or in the back with virus-containing growth medium of NIH 3T3 cells transformed by the following proviruses (see also Fig. 2): MoMLV, wild-type BalbSV (pB), recombinant BalbSV with normal proto-*ras*-specific codon 12 (pB12) and with a normal proto-*ras*-coding region (pBpral), wild-type HaSV (pA), and recombinant HaSV with normal proto-*ras*-derived codons 12 and 59 (pA1259) and with a normal proto-*ras*-coding region (pApralX). The amount of virus injected is indicated. Except for the MoMLV control, open circles indicate lethal erythroblastosis and solid circles indicate visible sarcoma formation at the site of injection.

specific codons (Fig. 4). Moreover, the lifespans of mice infected with wild-type and recombinant viruses were similar. All animals died or were moribund within about 60 days after inoculation. In all cases, erythroblastosis or fibrosarcoma was diagnosed by necropsy. A typical enlarged spleen with erythroblastic tumors of a mouse infected by a recombinant HaSV with normal proto-*ras*-specific codons (pApralX) is shown in Fig. 5. Control animals, inoculated with MoMLV helper virus only showed no signs of disease during the 60-day observation period. The proclivity of HaSV and BalbSV viruses to cause erythroblastosis rather than sarcomas at inoculation titers of 10^3 to 10^4 FFU confirms previous observations by others (10, 19, 22, 34, 39). It was concluded that wild-type and recombinant viruses containing normal proto-*ras*-coding regions were equally tumorigenic in mice under our assay conditions.

DISCUSSION

Our results clearly demonstrate that *ras* genes with viral or normal cellular coding regions under the control of a retro-

FIG. 5. Erythroblastic and enlarged spleen of a mouse infected with recombinant HaSV lacking virus-specific *ras* codons (pApralX). The spleen from a mouse 45 days after inoculation with erythroblastic tumors was about 3 cm long and 1.5 cm in diameter. The 3-day-old BALB/c mouse had been inoculated intraperitoneally with purified recombinant HaSV from a sucrose gradient (see Materials and Methods). pApralX is a recombinant HaSV with a normal proto-*ras*-coding region.

viral LTR promoter transform NIH 3T3 and diploid cells and cause tumors in animals. Thus, the transforming function of viral *onc* genes is not dependent on virus-specific point mutations in *ras* codon 12 or 59. This confirms and extends our previous conclusion (11) that the transforming function of viral *ras* genes is primarily due to truncation of cellular promoters and control elements and substitution with retroviral LTRs.

This conclusion is at variance with previous proposals by other researchers, who have proposed that point mutations confer transforming function to viral (4, 19, 27, 28, 39, 42) and cellular (3-6, 29, 40) *ras* genes. The proposal that *ras* genes derive their transforming function from specific point mutations was based on the observation that proto-*ras* genes with such mutations transform NIH 3T3 cells, while those without such mutations do not (29, 34, 40). However, subsequent work has shown that proto-*ras* genes with point mutations do not transform either rodent (23, 26, 31) or human (33) primary cells. It follows that the NIH 3T3 cell transformation assay fails to distinguish between *ras* genes that transform diploid cells and are thus potential cancer genes (16) and *ras* genes that transform only aneuploid NIH 3T3 cells. Hence, NIH 3T3 cell transformation is ambiguous as an assay for genuine cancer genes.

Although point mutations do not convert proto-*ras* genes to transforming genes, they do enhance transforming function and possibly the oncogenicity of *ras* genes that are under the control of retroviral promoters (LTRs) (11). This is particularly clear when transformation efficiencies of cloned *ras* DNAs are compared after transfection of proviral DNA in the absence of helper provirus. For example, LTR *ras* genes with point mutations generate about 10-fold more foci per microgram of DNA in NIH 3T3 cells than do those without (11). The reduced transforming efficiency of LTR *ras* genes without point mutations compared with that of those with mutations probably represents reduced transforming efficiency of unmutated p21^{ras} protein (18, 38). In addition, the specific focus-forming activity of provirus with proto-*ras*-coding sequences is about 10- to 30-fold lower and 3 to 6 times slower than that of viruses carrying spliced *ras*-coding regions, even in the presence of helper virus (Table 1). This difference is likely to reflect inefficient processing of unintegrated proviral DNA rather than an inherently lower transforming efficiency. Since the majority of transfected proviruses are never integrated into chromosomal DNA but are nevertheless transcribed to form infec-

tious transforming virus in the presence of helper virus (11), proviruses with unspliced *ras*-coding regions might be at a disadvantage compared with those with spliced coding regions. This is because RNA transcribed from unintegrated DNA might not be spliced and hence not be transforming and because unspliced RNA might not be packaged as efficiently as spliced RNA nor function as a template for reverse transcriptase as efficiently as spliced RNA. However, such RNA could nevertheless be infectious and would become transforming upon replication via an integrated provirus. In apparent contrast to the differences observed upon transfection with proviral DNAs, replicating viruses carrying *ras* genes with or without point mutations (relative to proto-*ras*) were indistinguishable in transformation assays with NIH 3T3 cells, diploid rat cells, or mice. Presumably, viral replication optimizes expression of viral genes and thus saturates the cell with *ras* protein. This high and optimal expression level of p21^{ras} in cells infected by viruses probably masks the difference between the transforming efficiencies of p21^{ras} proteins with and without mutations.

In light of our results, the generation of a retrovirus with a transforming *ras* gene can be envisioned as a multistep process. The first step would be a rare illegitimate recombination between a retrovirus and proto-*ras*, generating a transforming virus with a normal proto-*ras*-coding region. The transforming function of such a virus would be enhanced by truncation of noncoding proto-*ras* regions, such as proto-*ras* exon 1 and point mutation of the coding *ras* regions. These point mutations would readily appear upon selection for optimal transforming function in vivo and in vitro because of the high point mutation frequency of retroviruses (12, 41).

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LITERATURE CITED

- Andersen, P. R., S. R. Tronick, and S. A. Aaronson. 1981. Structural organization and biological activity of molecular clones of the integrated genome of a BALB/c mouse sarcoma virus. *J. Virol.* **40**:431-439.
- Balmain, A., M. Ramsden, G. T. Bowden, and J. Smith. 1984. Activation of the mouse cellular Harvey-*ras* gene in chemically induced benign skin papillomas. *Nature (London)* **304**:596-602.
- Barbacid, M. 1986. *ras* genes. *Annu. Rev. Biochem.* **56**:779-827.
- Bishop, J. M. 1983. Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* **52**:301-354.
- Bishop, J. M., and H. Varmus. 1984. Function and origins of retroviral transforming genes, p. 1058-1063. In R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *RNA tumor viruses*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Capon, D. J., E. Y. Chen, A. D. Levinson, P. H. Seeburg, and D. V. Goeddel. 1983. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature (London)* **302**:33-37.
- Chang, E. H., M. A. Gonda, R. W. Ellis, E. M. Scolnick, and D. R. Lowy. 1982. Human genome contains four genes homologous to transforming genes of Harvey and Kirsten murine sarcoma viruses. *Proc. Natl. Acad. Sci. USA* **79**:4848-4852.
- Chang, E. H., M. E. Furth, E. M. Scolnick, and D. R. Lowy. 1982. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. *Nature (London)* **297**:479-483.
- Chang, E. H., R. W. Ellis, E. M. Scolnick, and D. R. Lowy. 1980. Transformation by cloned Harvey murine sarcoma virus DNA: efficiency increased by long terminal repeat DNA. *Science* **210**:1249-1251.
- Chesterman, F. C., J. J. Harvey, R. R. Dourmashkin, and M. H. Salaman. 1966. The pathology of tumors and other lesions induced in rodents by virus derived from a rat with Moloney leukemia. *Cancer Res.* **26**:1759-1768.
- Cichutek, K., and P. H. Duesberg. 1986. Harvey *ras* genes transform without mutant codons, apparently activated by truncation of a 5' exon (exon-1). *Proc. Natl. Acad. Sci. USA* **83**:2340-2344.
- Coffin, J. M., P. N. Tsichlis, C. S. Barker, S. Voynow, and H. L. Robinson. 1980. Variation in avian retrovirus genomes. *Ann. N.Y. Acad. Sci.* **54**:410-425.
- DeFeo, D., M. A. Gonda, H. A. Young, E. H. Chang, D. R. Lowy, E. M. Scolnick, and R. W. Ellis. 1981. Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. *Proc. Natl. Acad. Sci. USA* **78**:3328-3332.
- Der, C. J., T. G. Krontiris, and G. M. Cooper. 1982. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the *ras* genes of Harvey and Kirsten sarcoma viruses. *Proc. Natl. Acad. Sci. USA* **79**:3637-3640.
- Dhar, K., R. W. Ellis, T. Y. Shih, S. Oroszlan, B. Shapiro, J. Maizel, D. Lowy, and E. Scolnick. 1982. Nucleotide sequence of the p21 transforming protein of Harvey sarcoma virus. *Science* **217**:934-937.
- Duesberg, P. H. 1987. Cancer genes: rare recombinants instead of activated cancer genes. *Proc. Natl. Acad. Sci. USA* **84**:2117-2124.
- Ellis, R. W., D. DeFeo, T. Y. Shih, M. A. Gonda, H. A. Young, N. Tsuchida, D. R. Lowy, and E. M. Scolnick. 1981. The p21 *src* genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. *Nature (London)* **292**:506-511.
- Feramisco, J. R., M. Gross, T. Kamata, M. Rosenberg, and R. W. Sweet. 1984. Microinjection of the oncogene form of the human H-*ras* (T24) protein results in rapid proliferation of quiescent cells. *Cell* **38**:109-117.
- Frederickson, T. N., R. R. O'Neill, R. A. Rutledge, T. S. Theodore, M. A. Martin, S. K. Ruscetti, J. B. Austin, and J. W. Hartley. 1987. Biologic and molecular characterization of two newly isolated *ras*-containing murine leukemia viruses. *J. Virol.* **61**:2109-2119.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
- Huang, A. L., M. C. Ostrowski, D. Bernard, and G. L. Hager. 1981. Glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from mouse mammary tumor virus. *Cell* **27**:245-255.
- Kirsten, W. H., and L. A. Mayer. 1967. Morphologic responses to a murine erythroblastosis virus. *J. Natl. Cancer Inst.* **39**:311-335.
- Land, H., L. F. Parada, and R. A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (London)* **304**:596-602.
- Langbeheim, H., T. Y. Shih, and E. M. Scolnick. 1980. Identification of a normal vertebrate cell protein related to the p21 *src* of Harvey murine sarcoma virus. *Virology* **106**:292-300.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Newbold, R. F., and R. W. Overell. 1983. Fibroblast immortality is a prerequisite for transformation by EJc-Ha-*ras* oncogene. *Nature (London)* **304**:648-651.
- Rasheed, S., G. L. Norman, and G. Heidecker. 1983. Nucleotide sequence of the Rasheed rat sarcoma virus oncogene: new

- mutations. *Science* **221**:155–157.
28. Reddy, E. P., D. Lipman, P. Anderson, S. R. Tronick, and S. A. Aaronson. 1985. Nucleotide sequence analysis of the BALB/c murine sarcoma virus transforming gene. *J. Virol.* **53**:984–987.
 29. Reddy, E. P., R. K. Reynolds, E. Santos, and M. Barbacid. 1982. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature (London)* **300**:149–152.
 30. Reynolds, S. H., S. J. Stowers, R. R. Maronpot, M. W. Anderson, and S. A. Aaronson. 1986. Detection and identification of activated oncogenes in spontaneously occurring benign and malignant hepatocellular tumors of the B6C3F1 mouse. *Proc. Natl. Acad. Sci. USA* **83**:33–37.
 31. Ruley, H. E. 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature (London)* **304**:602–606.
 32. Ruta, M., R. Wolford, R. Dhar, D. DeFeo-Jones, R. W. Ellis, and E. M. Scolnick. 1986. Nucleotide sequence of two rat cellular *ras*^H genes. *Mol. Cell. Biol.* **6**:1706–1710.
 33. Sager, R., K. Tanaka, C. C. Lau, Y. Ebina, and A. Anisowicz. 1983. Resistance of human cells to tumorigenesis by cloned transforming genes. *Proc. Natl. Acad. Sci. USA* **80**:7601–7605.
 34. Scher, C. D., E. M. Scolnick, and R. Siegler. 1975. Induction of erythroid leukaemia by Harvey and Kirsten sarcoma viruses. *Nature (London)* **256**:225–226.
 35. Shih, T. Y., M. D. Weeks, H. A. Young, and E. M. Scolnick. 1979. Identification of a sarcoma virus-coded phosphoprotein in non-producer cells transformed by Kirsten or Harvey sarcoma virus. *Virology* **96**:64–79.
 36. Shih, T. Y., M. D. Weeks, H. A. Young, and E. M. Scolnick. 1979. p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. *J. Virol.* **31**:546–556.
 37. Shoemaker, C., J. Hoffmann, S. P. Goff, and D. Baltimore. 1981. Intramolecular integration within Moloney murine leukemia virus DNA. *J. Virol.* **40**:164–172.
 38. Stacey, D. W., and H.-F. Kung. 1984. Transformation of NIH 3T3 cells by microinjection of Ha-*ras* p21 protein. *Nature (London)* **310**:508–511.
 39. Tabin, C. J., and R. A. Weinberg. 1985. Analysis of viral and somatic activations of the c-Ha-*ras* gene. *J. Virol.* **53**:260–265.
 40. Tabin, C. J., S. M. Bradley, C. I. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, D. R. Lowy, and E. H. Chang. 1982. Mechanism of activation of a human oncogene. *Nature (London)* **300**:143–149.
 41. Temin, H. M. 1988. Evolution of cancer genes as a mutation-driven process. *Cancer Res.* **48**:697–1701.
 42. Varmus, H. E. 1984. The molecular genetics of cellular oncogenes. *Annu. Rev. Genet.* **18**:553–612.