Analysis of Viral and Somatic Activations of the cHa-*ras* Gene

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The activation of the cHa-*ras* oncogene in the EJ/T24 bladder carcinoma cell line was compared with the activation of the same gene in the rat-derived Harvey murine sarcoma virus. The results indicate that, like the human oncogene, the Harvey murine sarcoma virus-borne *ras* gene owes its oncogenic capacity to point mutations in coding sequences rather than to the alteration in transcriptional control that occurred when the formerly cellular *ras* sequences were acquired by the virus. The viral gene retained its transforming ability when its transcription was removed from the influence of the retroviral long terminal repeat promoter and was placed under the regulation of the cHa-*ras* promoter. Conversely, the viral long terminal repeat was insufficient to activate the normal cHa-*ras* allele when a single copy of such a construct was delivered to a cell by viral infection. In addition to their mode of activation, the biological properties of the EJ/T24 and Harvey murine sarcoma virus oncogenes were compared by infecting newborn mice with chimeric retroviruses bearing each form. The two alleles acted equivalently, causing erythroleukemias and sarcomas with similar kinetics.

The cellular Ha-*ras* protooncogene has been activated into an oncogenic form via at least two routes. One route is through the creation of a hybrid sarcoma virus that involved both recombinational events with a retroviral genome (23, 28) and sequence alterations within the *ras* protein-encoding region (6). One example of such an activated Ha-*ras* allele is that borne by the Harvey murine sarcoma virus (HaSV) which arose in a rat (16). A second mode of Ha-*ras* activation depends on somatic mutational events having no apparent viral association. Such an activated form is present in the genome of the EJ/T24 human bladder carcinoma cell line (8, 18, 22). Although arising in distinct fashions, the two Ha-*ras* oncogenes of HaSV and EJ/T24 origin share a property in common: each carries a mutation in the codon specifying amino acid residue 12 of the 21,000-dalton protein known as p21 (3, 19, 29, 31). These mutations cause the glycine 12 residue specified by the Ha-*ras* protooncogenes to be replaced by either arginine or valine. In the case of the EJ/T24 allele, the amino acid 12 substitution has been shown to be sufficient to activate the gene (3, 19, 29, 31).

The above results might suggest that the activity of the two oncogenes was ultimately derived from the same type of molecular event. However, the HaSV oncogene differs from its EJ/T24 homolog in two other, potentially important respects. First, the viral oncogene encodes an additional amino acid substitution at residue 59 of the p21, causing the normally present alanine to be replaced by threonine (6). This replacement appears to be significant, as the identical replacement can be found in the p21 encoded by the independently arising Kirsten sarcoma virus (32). Second, the expression of the HaSV p21 is driven by the high-efficiency transcriptional promoter present in the long terminal repeat (LTR) of the viral genome (15). This contrasts with the regulation of the bladder carcinoma oncogene which continues to be controlled by the relatively weak promoter that regulates expression of the normal protooncogene. The high-level expression of the viral gene assumes some importance, since others have reported that the association of a nonmutated p21-encoding DNA segment with the LTR promoter is sufficient on its own to create an oncogene (2, 4, 7).

We wished to determine which aspects of the HaSV genome were indeed crucial for the activation of its oncogene and to determine whether the two Ha-*ras* oncogenes function similarly despite the above-mentioned differences.

MATERIALS AND METHODS

Mice. Untimed pregnant BALB/c mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Each female and subsequent litter was maintained in a separate cage throughout the experiments.

Virus stocks and cell lines. Moloney leukemia virus (MLV) was harvested from clone 1 cells constitutively producing virus (10). NIH 3T3 fibroblasts were maintained in Dulbecco modified Eagle medium containing 10% calf serum as described elsewhere (1). Virus stocks were harvested from producer cells 12 h after feeding. Infection of cells with MLV or MLV-pseudotyped virus was carried out in the presence of 8 μg of Polybrene (Aldrich Chemical Co., Inc. Milwaukee, Wis.) per ml for 2 h at 37°C, after the virus stocks were filtered through a 0.45-μm syringe filter. The titer of MLV virus was determined by the UV XC plaque assay (21). Titers of transforming virus were determined by infecting NIH 3T3 cells with various dilutions of virus and observing the number of foci arising after 10 to 14 days.

Cloned DNAs. pH-1 is a biologically active, circularly permuted form of the HaSV genome cloned in the *Eco*RI site in pBR322 (9). pH-1 was a gift from E. M. Scolnick. pEJ6.6 is a clone of the activated cHa-*ras* gene present in the EJ/T24 bladder carcinoma cell line cloned in the *Bam*HI site of pBR322 (24). pEC6.6 is a clone of the normal allele of cHa-*ras* cloned in the *Bam*HI site of pBR322 (29). pP3 is a similar normal cHa-*ras* clone obtained from M. Wigler and M. Goldfarb, which contains an *Mst*II site missing in the pEC6.6 clone (31). pZIP (termed p21 in reference 17) is an inactive proviral clone of MLV containing rat cellular flanking sequences cloned into the *Eco*RI site of a pBR322 vector that had previously been modified so as to remove its *Bam*HI site (17). pSV2Neo, a clone conferring resistance to G418 in mammalian cells, was a gift of R. C. Mulligan (27).

The HaSV coding region was placed under the control of the cHa-*ras* promoter by cutting pH-1 DNA with *Sac*I, removing the overhanging ends with the Klenow fragment of

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*Pol*I, and attaching *Eco*RI linkers. This clone was then cut with both *Eco*RI and *Bgl*II, and the resulting 1.1-kilobase (kb) fragment was purified by gel electrophoresis and NaI glass binding. The cHa-*ras* clone pP3 was digested with *Mst*II. The largest fragment was purified, *Eco*RI linkers were attached after the removal of overhanging ends, the DNA was cut with *Eco*RI and *Hin*dIII, and the smaller of the resulting fragments was isolated. This DNA piece was attached in a three-part ligation to the 1.1-kb pH-1 fragment and to a pUC13 vector clone which had been prepared by cutting with *Hin*dIII and *Bam*HI and treatment with calf intestinal phosphatase.

The pZIP MLV vector was modified by placing a *Bam*HI linker at the most 5' *Pst*I site in the MLV genome. pZIP was partially digested with *Pst*I, and singly cut molecules were isolated. Overhanging ends were filled in with DNA polymerase I, and *Bam*HI linkers were attached to the resulting ends. After *Bam*HI digestion and religation, a correctly modified vector was identified by virtue of its intact ampicillin resistance gene and the presence of only one *Pst*I site.

To construct chimeric virus genomes in which the inserted cHa-*ras* genes are under the viral transcriptional control, the pEC6.6 and pEJ6.6 plasmids were partially cut with *Mst*II, and the linear, singly cut fragments were isolated. These were then cut with *Sac*I, and the 2.6-kb fragments were purified. After the addition of *Bam*HI linkers, these were inserted into the modified pZIP *Bam*HI site. These viruses, carrying the EJ oncogene and the EC protooncogene were termed MLV(LTR)/EJ and MLV(LTR)/EC, respectively.

To produce chimeric virus genomes in which cHa-*ras* sequences remained under cellular transcriptional control, the pEJ6.6 plasmid was digested with *Bam*HI and *Cla*I. The 5.6-kb fragments were isolated. After the addition of *Bam*HI linkers, these were inserted into the modified pZIP *Bam*HI site. These plasmids carrying the EJ oncogene in two orientations were termed pEJV and pJEV.

Transfection of DNAs. Transfection of cloned transforming DNAs were carried out by CaPO₃ precipitation (1, 14) as previously described. Cloned DNA (10 ng) was used with 75 μg of carrier NIH 3T3 cell DNA. Foci were observed macroscopically in 10 to 15 days. Cotransfections were performed by precipitating 100 ng of nonselected DNA in a 20:1 molar ratio with pSV2Neo and 75 μg of NIH 3T3 DNA. On day 1 after transfection, the drug G418 was added at a concentration of 1 mg/ml. The selective medium was changed after 3 days. Colonies of resistant cells were observed macroscopically in 10 to 15 days.

Analysis of DNA and protein. The preparation of total cellular DNA, restriction enzyme digestion agarose gel electrophoresis, and transfer to nitrocellulose (26) were all carried out as previously described (13). ³²P-labeled probes were prepared by nick translation (20). Labeling of cells with [³⁵S]methionine, preparation of cell lysates, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were all carried out as previously described (29), except that 15% polyacrylamide was used instead of 12.5%. When very low levels of p21 were present, membranes rather than whole-cell lysates were prepared by homogenizing cells in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 7.1)–5 mM KCl–1 mM MgCl₂, followed by centrifugation for 5 min at 1,000 rpm. The supernatant was then centrifuged at 100,000 × *g* (35,000 rpm in an SW50.1 Beckman rotor) for 30 min. The pellets were suspended in the same buffer and floated on a 60% sucrose cushion for 5 min in an Eppendorf microcentrifuge. The floated membranes were then resuspended in lysis buffer (29) and treated

as whole-cell lysates, except that the cells of one confluent 150-cm tissue culture dish were used per lane. The monoclonal antiserum used in those experiments was Y13-259 (11), which detects both the HaSV-encoded p21 (25) and the human normal and transforming cellular cHa-*ras* protein (29).

Injection and autopsy of animals. One to two days after birth, mice were injected intraperitoneally with 0.1 ml of virus which had been filtered through a 0.45-μm filter. Each litter group received the same virus dilution. Litters ranged from 3 to 14 animals. One to three litters were injected with each dilution to a total of 8 to 14 mice. Animals were autopsied when they died, beginning 3 weeks postinjection, and remaining animals were sacrificed at 4 weeks. Mice were carefully dissected. Air-dried smears of blood and dissociated spleen cells were stained with hematoxylin and eosin and Giemsa stains.

RESULTS AND DISCUSSION

Mechanism of activation of the HaSV-associated oncogene. We first investigated whether the coding alterations in the HaSV-encoded p21 were sufficient to transform cells, independent of changes in the regulation of the gene. We attached the HaSV-borne oncogene to the cellular cHa-*ras* promoter (Fig. 1A). This recombinant was transfected into NIH 3T3 cells. In parallel, we transfected an unaltered clone of HaSV (pH-1; a kind gift from E. Scolnick [9]) as well as clones of the EJ bladder carcinoma transforming allele (pEJ6.6 [24]) and the normal nontransforming cellular Ha-*ras* allele (pEC6.6[29]). Whereas the normal cHa-*ras* clone did not produce any foci upon transfection, all of the remaining three constructs gave rise to foci at an efficiency of 5 × 10⁴ focus-forming units per μg of DNA. These foci
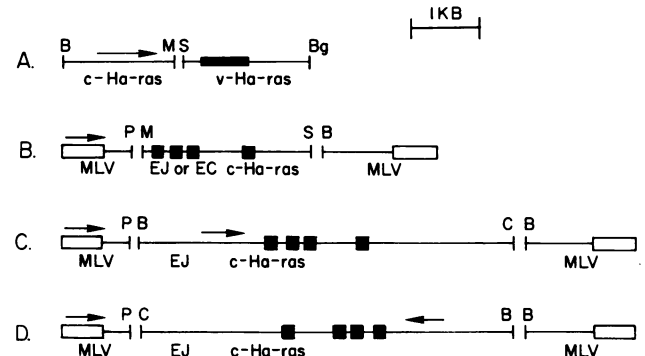

FIG. 1. Structure of constructs containing Ha-*ras* coding sequences. (A) The cHa-*ras* regulatory sequences used to drive the transcription of the HaSV-borne vHa-*ras* gene. (B) A transmissible retroviral vector carrying either the transforming (EJ) or normal (EC) cHa-*ras* coding sequences inserted so that the transcription of the *ras* genes is controlled by the high-level retroviral LTR promoter. (C) The EJ cHa-*ras* gene, including the cHa-*ras* transcriptional promoter and coding sequences, inserted into a transmissible retroviral vector in an orientation such that the cellular and viral LTR promoters initiate transcription in the same direction. (D) The same as in C, except that the insert is in the opposite orientation relative to the retroviral sequences. The following abbreviations are used for restriction sites: B, *Bam*HI; M, *Mst*II; S, *Sac*I; Bg, *Bgl*II; P, *Pst*I; and C, *Cla*I. Nonhomologous ends were joined with DNA linkers. Open boxes represent the retroviral LTR sequences, closed boxes represent *ras* gene coding exons, and arrows are used to indicate the approximate location of relevant transcriptional regulatory regions and the direction of transcription.

were all spindle shaped and refractile and displayed similar growth rates in soft agar. We concluded that the HaSV-encoded *ras* gene is able to transform cells without relying on any additional regulatory advantages provided by the strong retroviral promoter. This result suggested that HaSV might transform by the same mechanism as the cellular transforming allele in the EJ/T24 cell line.

These results bring into question the role of the highly efficient viral transcriptional promoter in the activation of the viral oncogene. Others have shown that this LTR promoter can activate an unmutated cHa-*ras* gene (4, 7). The resulting transforming activity was demonstrated in a transfection assay that delivers a high copy number of the transfected cloned gene into a recipient cell. We wished to see whether a single copy of the LTR-driven gene, as would be delivered by a virus, would also be adequate to transform a cell. To test this, we cut the cHa-*ras* normal and transforming alleles at an *Mst*II restriction site that is located 9 bases 5′ of the translational initiation codon AUG. These Ha-*ras* segments were then inserted into a DNA clone of MLV previously used as a retroviral vector (pZIP [17, 30]). The Ha-*ras* segments were inserted in such a way that we would expect transcription to be driven by the viral LTR (Fig. 1B). For convenience, we used a normal cHa-*ras* clone, pP3, kindly provided by M. Goldfarb and M. Wigler (31). This clone carried a sequence polymorphism that created a convenient restriction enzyme site. The resulting plasmids were referred to as pMLV(LTR)/EJ and pMLV(LTR)/EC.

Both the MLV(LTR)/EJ and MLV(LTR)/EC chimeric virus plasmids were cotransfected into NIH 3T3 cells with a simian virus 40-linked *neo* gene which confers resistance to the drug G418 (27). Drug-resistant colonies were then selected. All of the MLV(LTR)/EJ transfected colonies contained cells that showed strong morphological transformation. Some of the MLV(LTR)/EC colonies carried transformed cells, whereas others did not. [³⁵S]methionine-labeled whole-cell lysates were prepared from representative colonies of each type as well as from NIH 3T3 cells transfected with the simian virus 40 *neo* marker alone. These lysates were analyzed by immunoprecipitation with monoclonal antibodies and electrophoresis through a 15% polyacrylamide gel (Fig. 2). p21 was found to be present above the background level of NIH 3T3 cells (lane A) in those colonies transfected with MLV(LTR)/EJ (lane D) and in those nontransformed colonies transfected with MLV(LTR)/EC (lane C). p21 was present at very high levels in morphologically transformed cells transfected with MLV(LTR)/EC (lane B). The p21 encoded by the EJ allele has been previously shown to migrate with a slightly slower mobility than the normal variant (29). Thus, the two constructs induce synthesis of biologically active proteins which, to some extent, are capable of transforming cells in transfection.

We next attempted to passage the MLV(LTR)/EC and MLV(LTR)EJ constructs as viruses by superinfecting transfected cells with MLV. Of eight superinfected MLV(LTR)/EJ colonies, seven produced focus-forming supernatants with an average titer of 4 × 10³. In contrast, no foci were observed in the passage of virus rescued from 20 independent MLV(LTR)/EC lines.

Those foci which arose during passage of MLV(LTR)/EJ stocks represented the transmission of the expected virus as demonstrated by the presence of an altered form of p21 in infected cells. To visualize the p21 produced in picked foci of MLV(LTR)/EJ-infected cells, it was necessary to im-
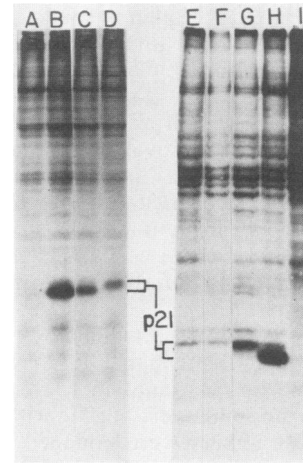

FIG. 2. Immunoprecipitation of ³⁵S-labeled p21 from cell lines transfected or infected with various constructs. Lanes A to D are from whole-cell lysates, and lanes E to I are from partially purified membrane preparations consisting of eight times the number of cells used in the first four lanes; thus, intensities of bands in the two panels cannot be directly compared. The brackets indicate the location of the EJ transforming variant of p21 (upper line) and the normal cellular p21 variant (lower line) in each lane. (A) NIH 3T3 cells. (B) NIH 3T3 cells transfected with MLV(LTR)/EC, the normal allele of cHa-*ras* in a retroviral vector utilizing the viral LTR promoter. These cells were morphologically transformed after transfection. (C) NIH 3T3 cells transfected with the same construct, but not transformed by it. (D) NIH 3T3 cells transfected with MLV(LTR)/EJ, the transforming EJ allele of cHa-*ras* in a retroviral vector utilizing the viral LTR promoter. (E) NIH 3T3 cells infected with EJV, a chimeric retrovirus carrying the cHa-*ras* EJ transforming gene, including the cellular regulatory sequences in the same orientation as the viral LTR. (F) NIH 3T3 cells infected with JEV, a chimeric retrovirus carrying the cHa-*ras* EJ transforming gene, including the cellular regulatory sequences in the opposite orientation from the viral LTR. (G) NIH 3T3 cells infected with MLV(LTR)/EJ, a chimeric retrovirus carrying the EJ cHa-*ras* coding sequences expressed off of the viral LTR promoter. (H) NIH 3T3 cells transfected with MLV(LTR)/EC and described in C, which had been subsequently infected with JEV. (I) NIH 3T3 cells. Immunoprecipitations were performed with an anti-p21 monoclonal antibody, Y13-259 (11), and resolved by 15% polyacrylmide gel electrophoresis.

munoprecipitate from isolated membranes rather than from whole-cell lysates. These membranes were prepared from approximately eight times as many cells as were used in the analysis of lysates of transfected cells. This alternate procedure was required because less protein is produced in an infected cell containing a single copy of the introduced *ras* gene than in a transfected cell which carries many acquired copies. This allowed p21 to be observed in EJ(LTR)/MLV cells (Fig. 2, lane G) in amounts well above the level seen in NIH 3T3 cells (lane I). We concluded that the LTR-driven normal cHa-*ras* allele is unable to transform cells when it is present in the low copy number resulting from viral infectious events.

The number of acquired Ha-*ras* sequences in these lines was investigated by preparing cellular DNA from morphologically transformed cells cotransfected with pMLV(LTR)/EJ, morphologically transformed cells cotransfected with pMLV(LTR)/EC, morphologically normal cells cotransfected with pMLV(LTR)/EC, and transformed cells resulting from infection with MLV(LTR)/EJ stocks. These

DNAs were analyzed by restriction enzyme cleavage, electrophoresis through a 0.75% agarose gel, and, after transfer to a nitrocellulose filter, by hybridization to an Ha-*ras*-specific DNA probe (Fig. 3). As expected, the DNA from transfectants contained a large number of Ha-*ras* hybridizing sequences (lanes A to C). The arrow indicates the approximate mobility of an 8.4-kb fragment. Since this is the size of the intact transfected and infected constructs, bands below the arrow mark reflect-fragmentary sequences. There was no obvious difference in the amount of intact Ha-*ras* hybridizing sequences in transformed (lane B) versus nontransformed (lane C) MLV(LTR)/EC cells; however, as shown above, the transformed MLV(LTR)/EC cells contained a greater amount of p21 protein product than did their nontransformed counterparts. In contrast to the transfected DNA, the infected DNAs of infected cells contained only a few acquired copies of the Ha-*ras* sequence (lane D). (The endogenous cellular Ha-*ras* sequences of mouse origin were not detected at this stringency of the hybridization with the human probe.)

These results suggest several conclusions. First, the removal of the normal Ha-*ras* gene from its chromosomal environment and its affiliation with a viral genome is not sufficient to impart oncogenicity. Second, LTR-controlled transcription is not per se sufficient to impart oncogenicity to a normal Ha-*ras*. Instead, the normal Ha-*ras* sequence can elicit transformation only when it is expressed in extremely high amounts, much higher than those created by a single copy of an LTR-driven gene. In the present case, such high amounts were achieved in the cells transfected with LTR-driven Ha-*ras* genes. These transformed cells acquired multiple copies of the viral genome as a consequence of the
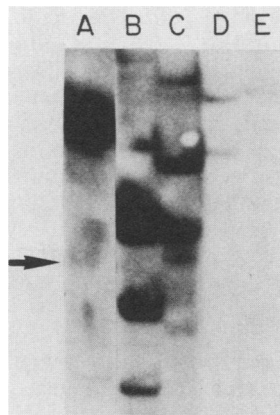

FIG. 3. Analysis of Ha-*ras* sequences present in cellular DNA after infection or transfection of various constructs. (A) NIH 3T3 cells which were morphologically transformed by transfection of pMLV(LTR)/EJ. (B) NIH 3T3 cells which were transformed by transfection with pMLV(LTR)/EC. (C) NIH 3T3 cells which were not morphologically transformed after transfection by pMLV(LTR)/EC. (D) NIH 3T3 cells transformed by infection with MLV(LTR)/EJ. (E) NIH 3T3 cells transformed by infection with JEV. DNAs were cut with the enzyme *Eco*RI which does not cut the MLV and *ras* sequences being analyzed, and after electrophoresis through 0.75% agarose and transfer to a nitrocellulose filter, the DNA was hybridized to a nick-translated human cHa-*ras* probe. Under the hybridization conditions used, the mouse homologous sequences were not visible. The arrow indicates the approximate mobility of an 8.4-kb fragment which would be the intact size of the infected and transfected constructs. Thus, bands below the arrow reflect fragmentary sequences.

transfection process. Infected cells, which acquired only one or several copies of the normal gene and linked LTR, were morphologically normal.

Mechanism of activation of the cellular oncogene. Because LTR-mediated regulation was not sufficient on its own to impart oncogenicity to the normal Ha-*ras* allele, we wondered whether the EJ oncogenic allele would be able to transform cells at the much lower level of expression achieved when driven by the weak cellular Ha-*ras* promoter and when delivered to cells in single copy. To this end, we constructed chimeric viruses in which a mutated *ras* oncogene remained under the control of the normal cellular Ha-*ras* transcriptional promoter. The promoter and coding regions of the genomic versions of the EJ/T24 bladder oncogene were isolated as a single 5.6-kb DNA fragment. This segment was introduced in either orientation into the pZIP retroviral vector (Fig. 1C and D). The resulting chimeric plasmids were termed pEJV (*ras* and LTR in the same transcriptional polarity) and pJEV (*ras* and LTR in the opposite polarity). Both were transfected into NIH 3T3 cells, and transformants were picked. Virus was rescued from both types of transformants by infection with MLV helper. Both virus stocks were able to induce foci. Several of these infected, transformed colonies were picked and used to create stable, virus-producing lines. Cells producing high-titer virus lines (5×10^6 focus-forming units per ml) were found with the EJV and JEV viruses.

The transformants created by the EJV and JEV viruses were indistinguishable morphologically. DNA was prepared from cells transformed by JEV infection. This DNA was analyzed by restriction enzyme cleavage, electrophoresis through a 0.75% agarose gel, and, after transfer to a nitrocellulose filter, by hybridization to an Ha-*ras* specific DNA probe. As expected, the RNA of infected cells contained only single acquired copies of the Ha-*ras* sequence (Fig. 3, lane E).

When membranes made from the EJV and JEV transformants were analyzed, they showed similar levels of p21 (Fig. 2, lanes E and F), much below the level found in cells infected with the MLV(LTR)/EJ virus (lane G), although clearly detectable above the p21 level immunoprecipitated from membranes of uninfected NIH 3T3 cells (lane I). The level is also below that of the p21 in the EJ/T24 bladder carcinoma cells, in which the Ha-*ras*-encoded protein can be easily visualized from precipitates of whole-cell lysates (29). Thus, association of the complete EJ/T24 oncogene with a viral LTR did not significantly increase its level of expression. This was true in both transcriptional orientations. Despite the low level of p21 induced by these constructed viruses, strong morphological transformation was observed. Therefore, the point mutation carried by an Ha-*ras* oncogene appears sufficient to impart oncogenicity, even when the oncogene is expressed from a weak transcriptional promoter and is introduced into the cell in low copy number.

Interaction of the oncogenic and normal Ha-*ras* alleles. The results of the viral infections showed that a single copy of the EJ Ha-*ras* oncogene could transform NIH 3T3 cells. Because these cells express their own endogenous, normal Ha-*ras* alleles, this suggested that the introduced oncogenes behaved in a dominant fashion. The reagents provided by the above-mentioned experiments allowed us to test this dominance directly. Specifically, we wished to determine whether oncogenic dominance would still be displayed in the presence of a large excess in the normal nontransforming gene and protein product. We utilized cells which had previously been transfected with the normal cHa-*ras* clone

driven off the retroviral LTR promoter and which appeared morphologically untransformed. We infected these with JEV virus. After infection, numerous transformed foci were observed. Such foci were picked and expanded. When lysates of these transformed cells were analyzed for immunoprecipitable p21 in their membranes (Fig. 2, lane H), a great excess of the normal p21 protein was seen relative to the more slowly migrating transforming protein (>20:1 ratio). This demonstrates the strength of the oncogenic allele over its normal counterpart.

Comparison of the biological effects of the viral and cellular oncogenes. Although the pEJV, pJEV, and HaSV were equally able to induce morphological transformation, it remained possible that other, more subtle phenotypic manifestations were affected by the remaining difference between the oncogenes, the altered residue 59 specified by HaSV. We thus assayed other biological effects of these various viruses by injecting them into 1- to 2-day-old BALB/c mice. A titer of HaSV comparable to the titers of JEV and EJV was obtained by dilution. All of the viral stocks exhibited an MLV helper virus titer of 10^6 in an XC assay.

Various dilutions (see Fig. 4) of each virus stock (EJV, JEV, and HaSV) were filtered through a 0.45-µm filter and inoculated intraperitoneally. Approximately 10 animals received each dilution of virus. Animals began to die after 3

weeks and were autopsied. All remaining animals were sacrificed at 4 weeks. All three transforming virus stocks (HSV, EJV, and JEV) gave rise to an erythroleukemic disease that was manifested by a grossly enlarged spleen which was readily palpable by week 3 after inoculation (Fig. 4A). In a number of animals, the enlarged spleen ruptured, causing death. This rupturing was observed in animals infected by any of the three viruses. The spleens contained both dark-colored nodules full of blood and pale, ill-defined foci previously described by others as being erythroblastic foci (5, 16). The foci induced by all three viruses were of similar appearance macroscopically and microscopically. All three transforming viruses also gave rise to small solid tumors (sarcomas) at a lower incidence than leukemias (Fig. 4B).

Similar titers of EJV, JEV, and HaSV were required to observe each disease. When the helper virus MLV was injected by itself, we observed no pathology during the time of the experiment. Tumors caused by each of the three transforming viruses were minced and applied to dishes with fresh medium. After 24 h, media were harvested, filtered, and used to infect NIH 3T3 cells. All three stocks gave rise to foci of the transformed cells, indicating that transforming virus was indeed present in each tumor.

We conclude that the two Ha-*ras* oncogenes behaved similarly in this test of biological activity despite their different modes of regulation, levels of expression, and encoded p21 proteins. It appears, therefore, that the mutation affecting residue 12 of the encoded protein may have been already sufficient to impart oncogenicity to HaSV. The other changes suffered by the viral Ha-*ras*, associated with an LTR and mutation at residue 59, would appear to be of secondary importance.

However, work in other laboratories, although substantiating the oncogenic potential created by the residue 12 valine substitution, has indicated that the amino acid 12 arginine substitution found in HaSV is only able to partially achieve this result in the absence of an additional alteration at residue 59 (D. Lowy, personal communication; E. Chang, personal communication). The arginine mutation would appear to be a weaker oncogenic allele in their hands, and the threonine 59 change is therefore functionally significant in conjunction with the arginine 12 substitution. The arginine alteration is also less potent than the valine change in recently reported biochemical experiments in which the valine substitution shows greater "up modulation" of viral p21 phosphorylation (12).

Our results indicate the functional equivalence of the cHa-*ras* gene altered by the single mutation found in the EJ/T24 bladder carcinoma cell line and the double mutation present in the homologous gene of HaSV. Additionally, we note the fact that an oncogene arising in a human bladder carcinoma is able to induce erythroleukemias and sarcomas in mice. This shows most dramatically the lack of tissue and species specificity exhibited by such activated *ras* oncogenes.
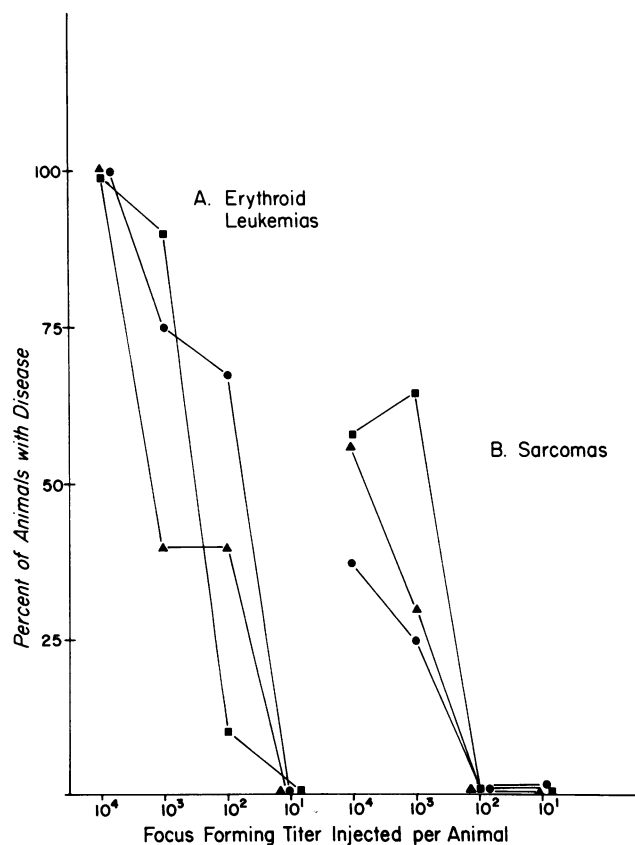

FIG. 4. Ability of Ha-*ras* viruses to cause malignant disease in newborn BALB/c mice. Approximately 10 mice were inoculated with 0.1 ml of each dilution of 0.45-µm filtered virus stock. Undiluted titers were 5×10^5 focus-forming units per ml in a focus assay on NIH 3T3 cells and 10^6 PFU/ml in an XC assay for the titer of helper virus (MLV). Mice were autopsied at death or after 4 weeks when they still survived. Symbols: ●, HaSV; ■, EJV; and ▲, JEV.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Andersson, P., M. P. Goldfarb, and R. A. Weinberg. 1979.** A defined subgenomic fragment of *in vitro* synthesized Moloney sarcoma virus DNA can induce cell transformation upon transfection. Cell **16:**63–75.
2. **Blair, D. G., M. Oskarsson, T. G. Wood, W. L. McClements, P. J. Fischinger, and G. G. Vande Woude. 1981.** Activation of the transforming potential of a normal cell sequence: a molecular model for oncogenesis. Science **212:**941–943.
3. **Capon, D. J., E. Y. Chen, A. D. Levinson, P. H. Seeburg, and D. V. Goeddel. 1983.** Complete nucleotide sequences of the T24 EJ human bladder carcinoma oncogene and its normal homologue. Nature (London) **302:**33–37.
4. **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.
5. **Chesterman, C., J. J. Harvey, R. A. 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.
6. **Dhar, R., 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 murine sarcoma virus. Science **217:**934–937.
7. **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 restriction endonuclease fragments homologous to the p21 coding region of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **78:**3328–3332.
8. **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. U.S.A. **79:**3636–3634.
9. **Ellis, R. W., D. DeFeo, J. M. Maryak, H. A. Young, T. Y. Shih, E. H. Chang, D. R. Lowy, and E. M. Scolnick. 1980.** Dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma virus. J. Virol. **36:**408–420.
10. **Fan, H., and M. Paskind. 1974.** Measurement of the sequence complexity of cloned Maloney murine leukemia virus 60 to 70*S* RNA: evidence for a haploid genome. J. Virol. **14:**421–429.
11. **Furth, M. E., L. J. Davis, B. Fleurdelys, and E. M. Scolnick. 1982.** Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular *ras* gene family. J. Virol. **43:**294–304.
12. **Gibbs, J. B., R. W. Ellis, and E. M. Scolnick. 1984.** Autophosphorylation of v-Ha-*ras* p21 is modulated by amino acid residue 12. Proc. Natl. Acad. Sci. U.S.A. **81:**2674–2678.
13. **Goff, S. P., O. N. Witte, E. Gilboa, N. Rosenberg, and D. Baltimore. 1981.** Genome structure of Abelson murine leukemia virus varients: proviruses in fibroblasts and lymphoid cells. J. Virol. **38:**460–468.
14. **Graham, F. L., and A. J. van der Eb. 1973.** A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–471.
15. **Hager, G. L., E. H. Chang, H. W. Chan, C. F. Garon, M. A. Israel, M. A. Martin, E. M. Scolnick, and D. R. Lowy. 1979.** Molecular cloning of the Harvey sarcoma virus closed circular DNA intermediates: initial structural and biological characteri-

16. **Harvey, J. J. 1964.** An unidentified virus which causes the rapid production of tumors in mice. Nature (London) **204:**1104–1105.
17. **Hoffmann, J. W., D. Steffen, J. Gusella, C. Tabin, S. Bird, D. Cowing, and R. A. Weinberg. 1982.** DNA methylation affecting the expression of murine leukemia proviruses. J. Virol. **44:**144–157.
18. **Parada, L. F., C. J. Tabin, C. Shih, and R. A. Weinberg. 1982.** Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus *ras* gene. Nature (London) **297:**474–479.
19. **Reddy, E. P., R. K. Reynolds, E. Santos, and M. Barbacid. 1982.** A point mutation is responsible for the acquisiton of transforming properties by the T24 human bladder carcinoma oncogene. Nature (London) **300:**149–152.
20. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977.** Labeling of DNA to high specific activity by nick translation. J. Mol. Biol. **113:**237–258.
21. **Rowe, W. P., W. E. Pugh, and J. Hartley. 1970.** Plaque assay techniques for murine leukemia virus. Virology **42:**1136–1139.
22. **Santos, E., S. R. Tronick, S. A. Aaronson, S. Pulciani, and M. Barbacid. 1982.** T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of Balb- and Harvey-MSV transforming genes. Nature (London) **298:**343–347.
23. **Scolnick, E. M., and W. P. Parks. 1974.** Harvey sarcoma virus: a second murine type C sarcoma virus with rat genetic information. J. Virol. **13:**1211–1219.
24. **Shih, C., and R. A. Weinberg. 1982.** Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell **29:**161–169.
25. **Shih, T. Y., M. O. Weeks, H. A. Young, and E. M. Scolnick. 1979.** Identification of sarcoma virus coded phosphoprotein in nonproducer cells transformed by Kirsten or Harvey murine sarcoma virus. Virology **96:**64–79.
26. **Southern, E. M. 1975.** Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
27. **Southern, P. J., and Berg, P. 1982.** Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1:**327–341.
28. **Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt. 1976.** DNA related to the tranforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature (London) **260:**170–173.
29. **Tabin, C. J., S. M. Bradley, C. F. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, D. Lowy, and E. H. Chang. 1982.** Mechanism of activation of a human oncogene. Nature (London) **300:**143–149.
30. **Tabin, C. J., J. W. Hoffmann, S. P. Goff, and R. A. Weinberg. 1982.** Adaptation of a retrovirus as a eucaryotic vector transmitting the herpes simplex virus thymidine kinase gene. Mol. Cell. Biol. **2:**426–436.
31. **Taparowsky, E., Y. Suard, O. Fasano, K. Shimizu, M. Goldfarb, and M. Wigler. 1982.** Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature (London) **300:**762–765.
32. **Tsuchida, N., T. Ryder, and E. Ohtsubo. 1982.** Nucleotide sequence of the oncogene encoding the p21 transforming protein of Kirsten murine sarcoma virus. Science **217:**937–939.

zation. J. Virol. **31:**795–809.