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Harvey Murine Sarcoma Virus: Influences of Coding and Noncoding Sequences on Cell Transformation In Vitro and Oncogenicity In Vivo

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The rat-derived Harvey murine sarcoma virus (Ha-MuSV) contains a transduced *ras* oncogene activated by two missense mutations and flanked by rat retroviruslike VL30 sequences. Ha-MuSV induces focal transformation of mouse NIH 3T3 cells in vitro and tumors (fibrosarcomas and splenic erythroleukemias) in newborn mice. We have used these two assays to study the contribution of coding and noncoding viral sequences to the biological activity of Ha-MuSV. A good correlation was found between the in vitro and in vivo assays. In several different isogenic Ha-MuSV variants, those with a ras^H gene that had one or both of the Ha-MuSV missense mutations were much more active biologically than the corresponding proto-oncogene. A Ha-MuSV variant that encoded the proto-oncogene protein induced lymphoid leukemias (with thymomas), with a relatively long latent period, rather than the fibrosarcomas and erythroleukemias characteristic of Ha-MuSV with one or both missense mutations. A VL30-derived segment with enhancer activity was identified downstream from v-ras^H. A mutant Ha-MuSV from which this 3′ noncoding segment was deleted expressed lower levels of the wild-type viral protein, displayed impaired transforming activity in vitro, and induced lymphoid leukemias (with thymomas). 5′ noncoding rat c-ras^H sequences were found to increase the biological activity of the virus when substituted for the corresponding segment of v-ras^H. We conclude that (i) the biological activity of Ha-MuSV can be influence significantly by noncoding sequences located outside the long terminal repeat as well as by coding sequences, (ii) VL30 sequences positively regulate the expression of v-ras^H, (iii) relatively low biological levels of *ras*, whether resulting from low-level expression of wild type v-ras^H or high-levels of *ras* proto-oncogene protein, induce a type of tumor that differs from tumors induced by high biological levels of *ras*, and (iv) the in vivo pathogencity of the Ha-MuSV variants correlated with their transforming activity on NIH 3T3 cells.

Harvey murine sarcoma virus (Ha-MuSV) is a rat-derived acute transforming retrovirus that carries a *ras* oncogene (v-ras^H; reviewed in references 1 and 20). Ha-MuSV efficiently induces focal transformation of NIH 3T3 cells in vitro and fibrosarcomas and splenic erythroleukemia in vivo. Although the biological activity of viruses often depends on both coding and noncoding sequences (reviewed in references 14, 18, and 26), the relative contribution of various viral elements has not been studied systematically for Ha-MuSV, which may account in part for the opposing conclusions that have been reached by different investigators about the biological significance of mutations within the coding sequences of v-ras^H.

Compared with the normal cellular ras^H proto-oncogene (c-ras^H), v-ras^H encodes two missense mutations. Analogous mutations in c-ras^H have been found in a variety of human and animal tumors (1). By using the NIH 3T3 cell transformation assay, it has been found that a *ras* gene carrying either or both of these mutations is more highly transforming than normal c-ras^H (16, 27). However, other data have been interpreted as suggesting that the mutations themselves contribute relatively little to the transforming activity of *ras* (7, 10). Controversy also exists about the biological significance of the NIH 3T3 cell transformation assay, especially with regard to *ras* (10, 19).

In this communication, we have made direct comparisons between focal transformation of NIH 3T3 cells and in vivo tumorigencity for wild-type Ha-MuSV and isogenic variants. The comparative analysis of well-defined Ha-MuSV variants has enabled us to characterize the influence of *ras*-coding sequences and certain noncoding viral sequences on the biological activity of the virus in vitro and in vivo, as well as to study the correlation between the two types of assays.

MATERIALS AND METHODS

Plasmid constructions. Plasmid contructions were performed by standard procedures (21). The plasmid pCO20-A contains the full-length Ha-MuSV genome derived from a cloned, circularly permuted Ha-MuSV DNA (clone H1) (11). It was generated by ligation of a 4.5-kilobase (kb) *Hin*dIII-*Eco*RI Ha-MuSV fragment (nucleotides [nt] 3495 to 2540 [30], which provided the 5′ long terminal repeat [LTR], packaging signals, and v-ras^H gene) to a 3.3-kb *Eco*RI-*Bam*HI Ha-MuSV fragment (nt 2540 to 378, which provided 3′ noncoding sequences and the 3′ LTR), inserted between the *Hin*dIII and *Bam*HI sites in pBR322. To generate clones expressing only one mutation (pCO21-S and pCO22-E; see Fig. 2) or no mutation (pCO23-W), Ha-MuSV sequences were replaced in pCO20-A by DNA fragments encoding the corresponding region of the normal cellular p21: the Ha-MuSV 51-base-pair (bp) *Hin*dIII-*Pvu*II (nt 1088 to 1139) fragment, which carries mutation 12, was replaced by the corresponding 51 bp from the normal mouse gene (15) in pCO21-E and pCO23-W; the Ha-MuSV 151-bp *Pvu*II-*Mst*I

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(nt 1139 to 1290), which carries mutation 59, was replaced by the corresponding normal 151 bp from the mouse BALB sarcoma virus (22). The influence of 5′ noncoding sequences on Ha-MuSV transformation was studied by making recombinants between the 5′ end of Ha-MuSV and the normal rat c-*ras*^{H}. The Ha-MuSV 5′ end *Sac*II-*Hin*dIII fragment spanning nt 940 to 1088 in pCO31-F, pCO33-M, pCO35-K, pCO20-A, and pCO22-E was substituted for 156 bp from the analogous region of c-*ras*^{H}, from *Eco*RI to *Hin*dIII. The viral *Sac*II site at nt 940 was replaced by an *Eco*RI linker (by B. M. Willumsen, University Institute of Microbiology, Copenhagen, Denmark). The resulting clones were called pCO30-F′, pCO32-H″, pCO34-L, pCO24-I′, and pCO25-J, respectively. In the study on the effect of 3′ noncoding sequences on Ha-MuSV transformation, pCO26-C was constructed by deleting, in pCO20-A, a *Pst*I-*Pst*I fragment spanning nt 1759 to 3436.

Cells, DNA transfection assay, and generation of viruses. NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin, 50 U/ml; streptomycin, 50 μg/ml). The DNA transfection procedure was described previously (34). As a selectable marker, a plasmid carrying the neomycin resistance gene (pSV2neo) (31) was cotransfected, in some experiments, with the *ras*-expressing plasmids. Neomycin selection was started 48 h after the transfection by adding 0.5 mg of G418 (Gibco) per ml to the medium. The procedure to generate viruses was described previously (33). An amphotropic helper virus (6) or Moloney murine leukemia virus (Mo-MuLV) (2) was used.

Immunoprecipitation, poly(A)$^+$ RNA dot blot, sequence analysis, and CAT assay. Metabolic labeling of infected NIH 3T3 cells with [^{35}S]methionine (250 μCi/ml) and p21 immunoprecipitation with a monoclonal antibody (Y13-238 or Y13-259) were carried out as described previously (35). Polyadenylated [poly(A)$^+$] RNA was purified and hybridized following standard procedures (21), with guanidinium isothiocyanate for cell lysis, ultracentrifugation through a cushion of cesium chloride for RNA isolation, and chromatography on oligo(dT)-cellulose for poly(A)$^+$ RNA purification. Sequence analysis was performed after subcloning of appropriate fragments into an M13 vector and dideoxy sequencing (25). The enhancer activity of DNA sequences was studied by inserting them in both orientations upstream and downstream from an enhancerless chloramphenicol acetyltransferase (CAT) vector (pA10-CAT) (17). The CAT activity expressed by each vector in NIH 3T3 and CV-1 cells was studied after transfection of 10 μg of plasmid DNA as described previously (13).

In vivo studies. In each experiment and for the different retroviral constructions, a similar amount of virus, as measured by focus-forming units, was inoculated intraperitoneally into inbred Swiss mice (ICFW and NFS). Similar results were obtained with each strain.

RESULTS

Ha-MuSV was isolated following multiple passages of Mo-MuLV in rats (12). The Ha-MuSV genome has a complex origin (Fig. 1). The 1-kb v-*ras*^{H} oncogene is derived from the rat c-*ras*^{H} proto-oncogene (9, 24). It is flanked by about 3.5 kb of rat-derived retroviruslike sequences called VL30 (or 30S) because these endogenous sequences, when expressed in rat cells, migrate as 30S RNA (reviewed in reference 8). A 1.5-kb sequence of Mo-MuLV-derived sequences form the viral long terminal repeats (LTRs) as well as sequences just upstream from the 3′ LTR. The v-*ras*^{H} oncogene, which encodes the only known Ha-MuSV protein product (p21), differs from its proto-oncogene in containing two missense mutations within its protein coding sequences (encoding Arg-12 for the normal Gly-12 and Thr-59 for the normal Ala-59) and in lacking intervening sequences. The importance of the LTR, which is the principal viral regulatory element, has been shown previously for cell transformation by v-*ras*^{H} (4). No functions have been specifically identified for the VL30-derived sequences in Ha-MuSV.

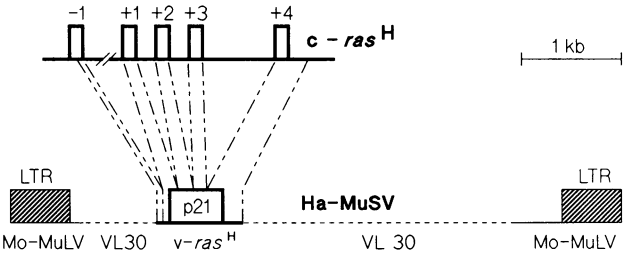
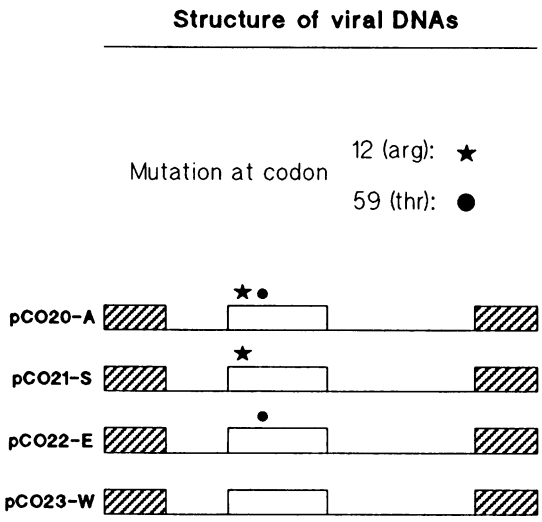


FIG. 1. Diagram of the 6-kb Ha-MuSV genome, with one LTR at both 5′ and 3′ ends as found in its provirus form. It is a complex recombinant between at least three different classes of sequences. The 1-kb v-*ras*^{H} oncogene and the five exons of the rat c-*ras*^{H} proto-oncogene from which v-*ras*^{H} is derived are represented as heavy solid lines. The open boxes correspond to the cellular and viral p21-coding sequences. The 3.5-kb flanking VL30 sequences are shown as dashed lines. Sequences derived from Mo-MuLV are thin solid lines; hatched boxes represent the LTRs.

In vitro and in vivo activity of Ha-MuSV variants. To study the influence of the v-*ras*^{H} missense mutations on the biology of Ha-MuSV, we constructed three isogenic variants of Ha-MuSV which differed from wild-type Ha-MuSV only at codons 12 and 59 (Fig. 2). pCO23-W encodes the normal c-*ras*^{H} proto-oncogene protein (Gly-12, Ala-59). It therefore permits an assessment of the biological potential of this normal protein in the context of the Ha-MuSV background. Single-amino-acid substitutions are encoded by pCO21-S (Arg-12) and pCO22-E (Thr-59). pCO20-A encodes the wild-type (Arg-12, Thr-59) viral protein.

Transfection of pCO23-W, encoding the normal protein, onto NIH 3T3 cells induced about 100 foci per μg of DNA (Fig. 2). Viral DNAs encoding either single missense mutation (pCO21-S and pCO22-E) or the wild-type v-*ras*^{H} (pCO20-A) induced foci at least 10 times more efficiently than pCO23-W. The foci induced by the mutants developed earlier and grew faster than those induced by the proto-oncogene (pCO23-W). When mass cultures of morphologically transformed cells were metabolically labeled with [^{35}S]methionine, immunoprecipitated with a *ras*-specific monoclonal antibody, and subjected to gel electrophoresis, *ras*-encoded protein bands of the expected migration rates, number, and intensity were obtained for each variant (Fig. 3). Significantly more p21 protein was detected in cells transformed by the proto-oncogene (pCO23-W), consistent with its lower transforming potential. The DNAs encoding Arg-12 (pCO21-S and pCO20-A) migrated somewhat more slowly than the normal protein. The two genes encoding Thr-59 (pCO22-E and pCO20-A) formed a doublet, since a proportion of p21 proteins containing Thr-59 are known to be autophosphorylated posttranslationally at this residue (28). These results are consistent with each variant's encoding its predicted protein product.



	In vitro		In vivo					
			mean survival (SEM), in days		tumor Induction			
			virus dilution injected					
	foci / ug DNA (x1000)	virus titer (ffu/ml)	10^0	10^{-2}	virus dilution	fibrosarcoma	erythroleukemia	lymphoid leukemia
pCO20-A	7	10^8	8(3)	29(9)	10^{-2}	34/34	34/34	0/34
pCO21-S	3	10^8	ND	38(8)	10^{-2}	8/8	6/8	1/8
pCO22-E	2	10^8	ND	35(7)	10^{-2}	7/7	5/7	1/7
pCO23-W	0.1	10^6	60(12)	ND	10^0	0/12	2/12	10/12

FIG. 2. Transforming activity in vitro and tumorigenicity in vivo induced by Ha-MuSV variants. pCO20-A is the wild-type viral DNA, containing the two v-*ras*^{H} mutations. The isogenic variants are pCO23-W, which encodes the normal protein of c-*ras*^{H}, and pCO21-S and pCO22-E, which contain the codon 12 and codon 59 mutations, respectively. Fibrosarcomas were located in the diaphragm. Mice with erythroleukemia had very large spleens. All mice with lymphoid leukemia had enlarged lymph nodes and no splenomegaly, and about half of them had thymomas. Open boxes, p21-coding sequences; hatched boxes, LTRs. ND, Not done. For tumor induction, numbers represent mice with tumors/number tested.

Superinfection of the transformed cells with a helper virus (amphotrophic MuLV or Mo-MuLV) efficiently pseudotyped the Ha-MuSV variants, enabling them to be transmitted as viruses to uninfected cells. Although the virus preparation encoding the normal protein contained more viral RNA than the other three virus preparations (data not shown), the titer of the focus-forming activity of the proto-oncogene-containing virus (pCO23-W) was, as expected, lower (by about 2 orders of magnitude) than that of the preparations with the mutant genes (Fig. 2); the foci induced by the proto-oncogene virus (derived from pCO23-W) also arose later, as had also been found for the corresponding viral DNA.

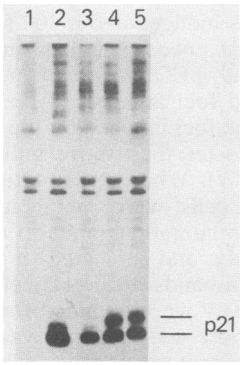
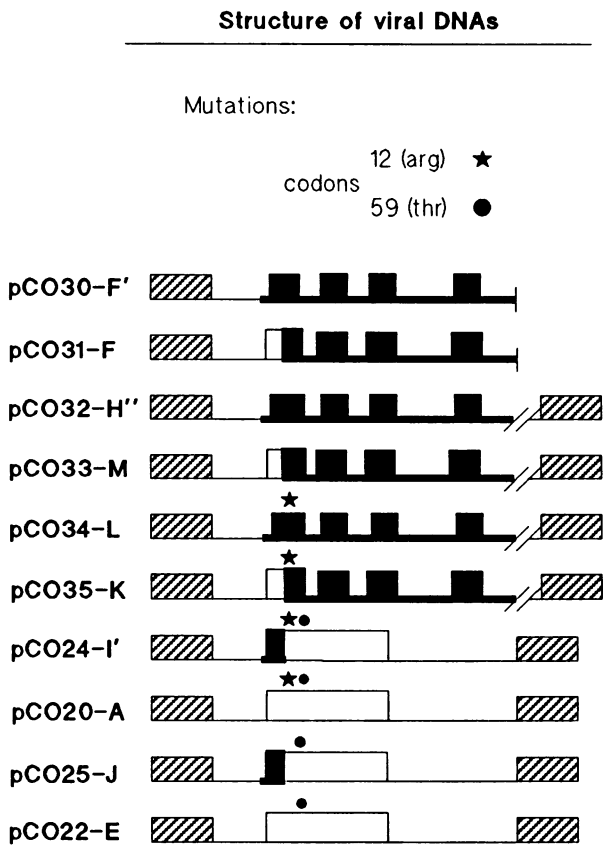


FIG. 3. Immunoprecipitation of p21 *ras*^{H} protein. [^{35}S]methionine-labeled cell extracts were immunoprecipitated with a *ras*-specific monoclonal antibody (Y13-259) from mass cultures of NIH 3T3 cells contransfected with pSV2neo and career DNA alone as a control (lane 1), plasmid DNAs pCO23-W (no mutation ["proto-oncogene"], lane 2), pCO21-S (mutation Arg-12, lane 3), pCO22-E (mutation Thr-59, lane 4), and wild-type pCO20-A (mutations Arg-12 and Thr-59 [authentic viral oncogene], lane 5). The upper p21 band in lanes 4 and 5 represents the phosphorylated form of the protein (on Thr-59).

The Ha-MuSV variants pseudotyped with amphotropic MuLV were then injected intraperitoneally into newborn inbred swiss mice (Fig. 2). As expected, wild-type Ha-MuSV (derived from pCO20-A) induced disease with a very short latency. High doses of virus were lethal to the mice in only 8 days; the animals apparently died from cardiopulmonary problems. Smaller inocula of virus prolonged survival of the mice; animals that succumbed 3 to 6 weeks after virus inoculation were found to have very large spleens (splenic erythroleukemia) and multiple fibrosarcomas, most of which were diaphragmatic. Similar results were obtained with the variants containing the single missense mutations (derived from pCO21-S and pCO22-E).

The virus derived from pCO23-W, encoding the normal protein, was also found to induce tumors. Compared with the viruses encoding the missense mutations, the proto-oncogene-containing virus was less pathogenic and induced a different type of tumor. The animals given the proto-oncogene virus (derived from pCO23-W) survived twice as long as those given a similar number of focus-forming units of a virus encoding one or both missense mutations. The tumors that developed in animals inoculated with the proto-oncogene virus were lymphoid leukemias (with thymomas), rather than the splenic and fibroblastic tumors that arose in animals inoculated with the viruses encoding missense mutations. As expected, the amphotropic helper virus by itself did not induce disease, even after 8 months.

The consistent formation of a different tumor type by pCO23-W-derived virus suggested that the tumors had not arisen via a mutation analogous to those present in the other three virus preparations. This hypothesis was confirmed by characterization of the virus isolated from a thymoma induced with pCO23-W-derived virus. It was found to induce foci on NIH 3T3 cells at the same relatively slow rate as the orginial pCO23-W-derived virus, to induce lymphoid leukemias (with thymomas) with a latent period similar to that of pCO23-W-derived virus, and to encode a *ras* protein on



	In vitro		In vivo				
			tumor Induction				
	foci / ug DNA (x1000)	virus titer (ffu/ml)	virus dilution	mean survival (SEM), in days	fibrosarcoma	erythroleukemia	lymphoid leukemia
pCO30-F'	0.05–0.1	–	–	–	–	–	–
pCO31-F	<0.01	–	–	–	–	–	–
pCO32-H''	0.02–0.2	10^5	ND	ND	ND	ND	ND
pCO33-M	<0.01	ND	ND	ND	ND	ND	ND
pCO34-L	3–10	10^6	10^0	60(12)	13/13	2/13	0/13
pCO35-K	0.5–2	ND	ND	ND	ND	ND	ND
pCO24-I'	5–15	10^9	10^{-3}	14(2)	17/18	18/18	0/18
pCO20-A	3–10	10^8	10^{-2}	29(9)	34/34	34/34	0/34
pCO25-J	5–15	10^9	10^{-3}	14(5)	12/14	14/14	0/14
pCO22-E	1–3	10^8	10^{-2}	35(7)	7/7	5/7	1/7

FIG. 4. Influence of 5' noncoding sequences on c-*ras*^{H} and v-*ras*^{H} in vitro transforming activity and in vivo tumorigenicity. A 148-bp fragment at the 5' end of v-*ras*^{H} (the *Sac*II-*Hin*dIII fragment spanning nt 940 to 1088 [30]) was substituted for a 166-bp fragment from the analogous region of c-*ras*^{H} (*Eco*RI-*Hin*dIII fragment), after replacement of the viral *Sac*II site (nt 940) by an *Eco*RI linker. The common *Hin*dIII site, which marks the 3' end of these two fragments, is located at codon 5 (of each gene). Thin lines and open boxes, Viral sequences; heavy lines and black boxes, rat cellular sequences; hatched boxes, LTRs. See Fig. 2 legend for other details.

sodium dodecyl sulfate gels that was indistinguishable from that of pCO23-W-derived virus (data not shown).

We conclude that in this construction, the *ras*^{H} proto-oncogene can be oncogenic for mice, is less transforming in vitro and less oncogenic in vivo than isogenic variants encoding the missense mutations tested (Arg-12 or Thr-59), and induces tumors in a different tissue than those induced by viruses carrying the missense mutations.

Influence of 5' noncoding sequences on Ha-MuSV transformation. The above experiments indicate that minor alterations in *ras*-coding sequences by themselves can have important consequences for the oncogenicity of Ha-MuSV. As described below, we also noted that sequences located upstream and downstream from the *ras*-coding sequences can influence transforming activity in vitro and oncogenicity in vivo.

The possible significance of sequences located 5' of the coding sequences was first suggested when we compared the transforming activity of two DNAs which contained the coding exons of the rat c-*ras*^{H} proto-oncogene. The 2.3-kb rat c-*ras*^{H} genomic *Eco*RI-*Bam*HI fragment that was studied contains all four coding exons as well as 154 nt of intron sequences located just upstream from the first coding exon. The 5' noncoding exon (−1) of c-*ras*^{H} is not present in this genomic fragment, since the genomic sequences that give rise to this exon are located further upstream (Fig. 1) (7, 9).

In the initial construction, a 1.7-kb Ha-MuSV fragment, composed of the LTR and 5' sequences, was fused to a 2.1-kb c-*ras*^{H} fragment via a *Hin*dIII site (at nt 1088) in Ha-MuSV that is common to the coding sequences of c-*ras*^{H} and v-*ras*^{H} (pCO31-F, Fig. 4). The protein encoded by this chimeric gene is identical to that encoded by the c-*ras*^{H} proto-oncogene, since the missense mutations in v-*ras*^{H} are located downstream from the *Hin*dIII site (which is at codon 5). The other clone (pCO30-F') was also a recombinant between the 5' end of Ha-MuSV and c-*ras*^{H}. Clone F' differed from clone F in that its Ha-MuSV-derived sequences were 148 nt shorter, extending only to the viral *Sac*II site at nt 940, while its c-*ras*^{H} sequences were 166 nt longer, being composed of the entire 2.3-kb c-*ras*^{H} fragment. Although pCO31-F and pCO30-F' each encoded the same *ras* protein, the transforming activity of pCO30-F' on NIH 3T3 cells was found to be at least 10 times greater than that of pCO31-F. Indeed, pCO31-F failed to induce focal transformation on NIH 3T3 cells under the conditions tested.

The influence of these 5' noncoding sequences was then studied in several rescuable isogenic viral DNA constructions that encoded either the proto-oncogene protein or the v-*ras*^{H} missense mutations (Fig. 4). As was true of clones pCO30-F' and pCO31-F, these constructions were used to compare the effect of substituting the first 148 bp at the 5' end of v-*ras*^{H} (the *Sac*II-*Hin*dIII fragment spanning nt 940 to 1088) for the 166 bp from the analogous region of c-*ras*^{H} (from *Eco*RI to *Hin*dIII). As noted above, the nucleotide

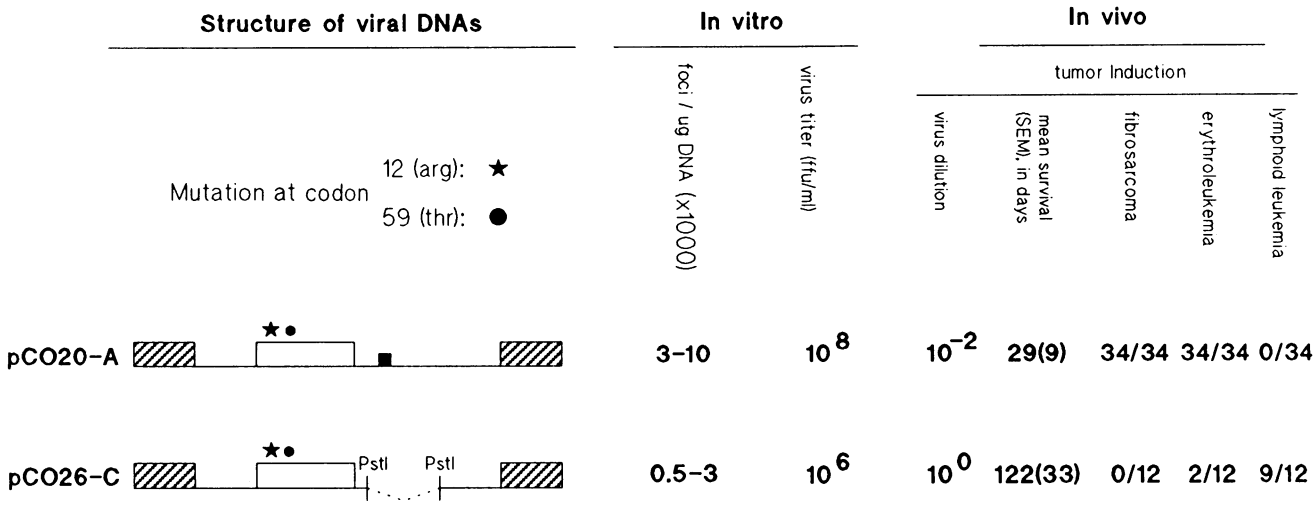


FIG. 5. Influence of 3′ noncoding sequences on v-*ras*^{H} in vitro transforming activity and in vivo tumorigenicity. Deletion of a fragment spanning nt 1759 to 3436 from wild-type Ha-MuSV (pCO20-A) resulted in low-transforming-ability viral DNA (pCO26-C). The deleted sequences were shown to contain an enhancer element. The core enhancer was located at nt 2101 to 2108 (black square). Open boxes, p21-coding sequences; hatched boxes, LTRs. See Fig. 2 legend for other details.

changes resulting from this substitution are limited to the 5′ noncoding sequences. This substitution was made for c-*ras*^{H} constructions encoding the Gly-12/Ala-59 proto-oncogene (pCO32-H″ vs. pCO33-M), for c-*ras*^{H} encoding the Arg-12 mutation (pCO34-L vs. pCO35-K), for wild-type Ha-MuSV (pCO24-I′ vs. pCO20-A), and for the Gly-12/Thr-59 mutant (pCO25-J vs. pCO22-E).

Significant and consistent differences in transforming activity on NIH 3T3 cells were noted when 5′ noncoding sequences from c-*ras*^{H} were substituted for noncoding v-*ras*^{H} sequences as described above (Fig. 4). For each isogenic comparison, the presence of c-*ras*^{H} 5′ noncoding sequences led to more efficient transformation by viral DNA (by a factor of 2 to 10) and to a higher titer of virus recovered (by about 1 order of magnitude). As had been true for the Ha-MuSV variants described in the previous section, there was relatively little difference, in isogenic constructions, between the transforming activity of *ras* carrying one or both missense mutations (compare pCO24-I′ and pCO25-J).

The higher transforming activity noted in vitro for pCO24-I′ versus pCO20-A (wild-type Ha-MuSV) and pCO25-J versus pCO22-E was correlated with their being more pathogenic in vivo. The pCO24-I′- and pCO25-J-derived viruses induced tumors that were similar to those induced by authentic Ha-MuSV, but the animals died sooner than those inoculated with virus derived from pCO20-A and pCO22-E.

3′ Noncoding sequences affect Ha-MuSV. Sequences located downstream from v-*ras*^{H} were also found to influence the biological activity of Ha-MuSV (Fig. 5). A clone (pCO26-C) was constructed whose only difference from the wild type was that it carried a 1.7-kb deletion between two *Pst*I sites located downstream from the v-*ras*^{H} coding sequences. Except for the first 78 nt, the deleted segment was composed of 30S-derived sequences. The virus derived from this clone was found to be less transforming in vitro and less oncogenic in vivo than wild-type Ha-MuSV, although pCO26-C DNA and its rescued virus encoded the authentic v-*ras*^{H} protein (Fig. 5).

When transfected into NIH 3T3 cells, this deletion mutant DNA induced transformed foci about 1 order of magnitude less efficiently than wild-type viral DNA (Fig. 5). The foci induced by pCO26-C developed 2 to 4 days later and grew more slowly than those induced by the wild type (pCO20-A). The titer of the virus recovered from these transfected cells by amphotropic virus superinfection was about 2 orders of magnitude lower than that of the wild type. Inoculation of newborns with this virus led, after a relatively long latent period (about 4 months), to the development of lymphoid leukemias (with thymomas) that were similar to those induced by pCO23-W-derived virus containing the proto-oncogene. After 8 months, no disease was observed in newborns inoculated with the amphotropic helper virus. These results indicate that sequences within the 1.7-kb 3′ noncoding fragment exert a positive influence on the biological activity of Ha-MuSV.

Analysis of 3′ noncoding sequences. To study the potential role of the 1.7 kb that were deleted in pCO26-C, viral RNA expression and *ras* protein levels were compared for cells transformed by virus derived from pCO26-C and from wild-type Ha-MuSV (pCO20-A). Both viral RNA and p21 protein were found to be reduced 5- to 10-fold in pCO26-C virus (Fig. 6). These results suggested that sequences within the deleted 1.7-kb *Pst*I fragment contributed to expression of the viral DNA.

To further localize the 3′ noncoding sequences that positively regulated Ha-MuSV, the in vitro transforming activity of viral DNAs that contained the LTR, v-*ras*^{H}, and progressively larger portions of the 1.7-kb 3′ noncoding segment deleted in pCO26-C was tested. A plasmid whose viral DNA sequences terminated at the *Pst*I site (nt 1759) marking the 5′ end of the deletion displayed low transforming activity on NIH 3T3 cells (500 FFU/μg of DNA), analogous to that seen with pCO26-C; similar low transformation activity was obtained when the viral DNA fragment was extended an additional 264 nt downstream to an *Xba*I site at nt 2023. In contrast, extending the viral DNA to a *Hin*cII site at nt 2168 resulted in virtually full transforming activity (7,000 FFU/μg of DNA); similar high transforming activity was obtained with a fragment extending to the *Eco*RI site at nt 2540.

These results suggested that sequences critical for increased transforming activity were located within the 145-nt *Xba*I-*Hin*cII fragment (nt 2023 to 2168), which is composed entirely of VL30-derived sequences. Inspection of the sequence in this fragment indicated that it contained a potential

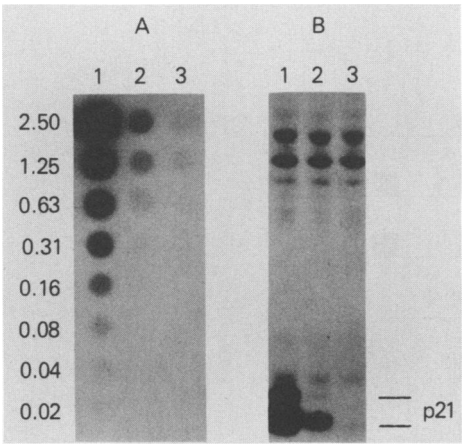


FIG. 6. Viral RNA dot blot and p21 immunoprecipitation from NIH 3T3 cells chronically infected with wild-type Ha-MuSV (pCO20-A, lanes 1) or with the VL30 deletion mutant (pCO26-C, lanes 2). Cells infected with the helper amphotropic virus alone was used as a control (lanes 3). (A) Dot blot with serial dilutions of RNA. Total amount (in micrograms) of poly(A)$^+$ RNA are indicated for each blot. The RNA was hybridized with a probe specific for Ha-MuSV (spanning VL30 sequences downstream from v-*ras*^{H} [23]). (B) p21 immunoprecipitation of [^{35}S]methionine-labeled cell extracts with a specific monoclonal antibody (Y13-259). The upper p21 band in lanes 1 and 2 of panel B is the phosphorylated form of the protein.

core enhancer element (GAGGAAAG at nt 2101 to 2108; Fig. 7). This sequence falls in a part of the viral DNA that is homologous to p30^{gag} of Mo-MuLV, although insertions and deletions in VL30 would prevent it from encoding a functional protein. The Mo-MuLV sequence, however, does not possess this potential enhancer sequence. Our sequence analysis of the analogous region in Kirsten (Ki) MuSV (*Pvu*II-*Hin*cII, about 1 kb downstream from v-*ras*^{K} [32]), which also contains 30S-derived sequences, indicated that Ki-MuSV possesses the same potential core enhancer element (GAGGAAAG). In fact, the VL30-derived sequences spanning nt 1832 to 2168, which are immediately downstream from v-*ras*^{H}, are identical in Ki-MuSV [except that Ki-MuSV has two more A nucleotides in a poly(A) run that, in Ha-MuSV, spans nt 1957 to 1963].

To examine the possibility that these 30S sequences might possess enhancer activity, we inserted a 207-bp *Stu*I-*Hin*cII fragment (nt 1963 to 2168), which is composed entirely of VL30-derived sequences, in both orientations upstream and downstream from an enhancerless CAT vector with a simian virus 40 (SV40) promoter (pA10-CAT) and determined the CAT activity of these constructions in CV-1 cells (Fig. 8). A modest (7- to 10-fold) but reproducible increase in CAT activity was noted for each construction. We conclude that this region contains an enhancer element.

DISCUSSION

In this report, we have systematically studied the biological activity in vitro and in vivo of defined Ha-MuSV variants. A good correlation was found between NIH 3T3 cell-transforming activity in vitro and viral tumorigenicity in vivo. A virus encoding the normal c-*ras*^{H} proto-oncogene protein in place of v-*ras*^{H} possessed a low level of oncogenicity, isogenic viruses carrying either one or both missense mutations contained in v-*ras*^{H} were highly oncogenic, non-LTR sequences located upstream and downstream from the v-*ras*^{H}-coding region could have a substantial influence on the oncogenicity of the virus, and the class of tumor induced in vivo correlated with the transforming activity in vitro. Our results indicate that the biological activity of the virus depends on many sequences within the virus rather than just on the presence or absence of missense mutations in *ras*. It seems likely that investigation of other viral sequences with no known function would reveal that they also contribute to the pathogenicity of the virus.

To analyze the contribution of the two missense mutations in v-*ras*^{H}, we compared wild-type Ha-MuSV with isogenic variants that carry only one of the v-*ras*^{H} mutations or that encode the same protein product as the proto-oncogene. Our results, which confirm and extend those of others, demonstrate that either missense mutation by itself markedly enhances the transforming activity of viral DNA or virus on NIH 3T3 cells and significantly increases the oncogenicity of the virus in vivo (16, 27). Wild-type v-*ras*^{H} may be somewhat more pathogenic than v-*ras*^{H} containing only one of the two missense mutations, but the differences were marginal under the conditions tested.

We also found that the Ha-MuSV variant encoding the proto-oncogene protein was tumorigenic. The in vivo activity of this virus differed from that of wild-type virus in two important respects. The mean survival time for animals inoculated with the proto-oncogene virus was significantly longer, and it induced lymphoid leukemia (with thymoma), in contrast to the splenic erythroleukemia and fibrosarcomas induced by wild-type Ha-MuSV. The tumors induced by the proto-oncogene do not appear to have arisen by virtue of an

```
Mo-MuLV    CTG TTG GGG ACT CTG CTG ACC GGA GAA GAA AAA CAA CGG GTG
           Leu Leu Gly Thr Leu Leu Thr Gly Glu Glu Lys Gln Arg Val
           ||| |||          ||| ||| |||     ||| |||     |||
           Leu Leu Gln      Leu Leu Thr Thr Glu Glu Arg Gln     Ile
Ha-MuSV    CTG TTA CAG A T CTT CTTTACC ACT GAG GAA AGA CAG  A  ATC
                                          core enhancer

Mo-MuLV    CTC TTA GAG GCT AGA AAG GCG GTG CGG GGC GAT GAT GGG CGC
           Leu Leu Glu Ala Arg Lys Ala Val Arg Gly Asp Asp Gly Arg
           ||| |||     ||| ||| |||     |||             ||| ||| |||
           Leu Leu Lys Ala Arg Lys Asn Val Pro Asp Met Asp Gly Arg
Ha-MuSV    CTG CTA AAG GCC AGA AAG AAT GTT CCA GAC ATG GAT GGA AGG
```

FIG. 7. Comparison between Mo-MuLV p30^{gag} sequences (nt 1437 to 1520 [29]) and Ha-MuSV VL30 sequences (nt 2076 to 2158 [30]), which increase the transforming activity and contain a core enhancer. The sequences have been aligned to emphasize their homology.

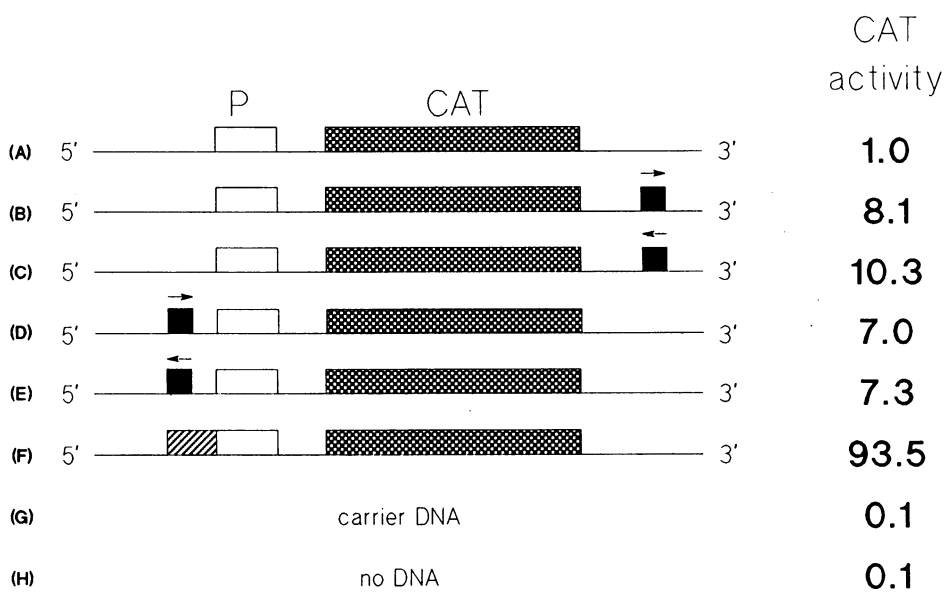


FIG. 8. Enhancer activity of VL30 sequences located downstream from v-*ras*^{H}. A fragment spanning nt 1963 to 2168 and containing the core enhancer (black box) was inserted in both orientations (arrows) downstream and upstream (B to E) from a CAT (cross-hatched box) vector, pA10-CAT (A). This enhancerless vector contains only the SV40 TATA element and 21-bp repeats (open box). The relative CAT activity, measured in CV-1 cells after transfection, is given as the percent chloramphenicol converted to acetylated forms relative to the percent converted by the pA10-CAT control (A). Positive control: SV40 promoter with its enhancer (72-bp repeats, hatched box) (F). Negative control: transfection assay with carrier DNA alone (G) and no DNA (H).

activating mutation in the virus, since reinjection of virus isolated from a thymic tumor also induced lymphoid leukemia and thymoma with a long latency. Chang et al. found, in their studies of a *ras*^{H} proto-oncogene-containing virus, that it failed to induce disease under the conditions tested (5). Their virus constructions and mouse strains differed somewhat from those used here, which may account for the nononcogenic behavior of the proto-oncogene virus in their studies. Cichutek and Duesberg reported tumor formation by a virus whose oncogene was derived from c-*ras*^{H} (7).

The results show further that VL30-derived sequences located downstream from v-*ras*^{H} positively affect the transforming activity of the virus. A Ha-MuSV variant that is missing these sequences, although it encodes the wild-type v-*ras*^{H} protein, induces lymphoid leukemias (with thymomas) after a much longer latent period than wild-type Ha-MuSV. The lower transforming activity and RNA expression of the deletion mutant were noted in the viral DNA transfection, which indicates that the lower biological activity of the virus carrying this deletion cannot be attributed to inefficient pseudotyping as a result of the deletion. Our studies strongly suggest that the lower level of expression results from the presence of an enhancer element within the deleted sequences. From sequence analysis, it is apparent that Ki-MuSV also contains this element. The apparent contribution of the VL30 sequences to the titer of the virus and to the pathological activity of both viruses provides a potential explanation for the transduction of these sequences during formation of the viruses. The presence of an enhancer element in the *gag* sequences of an avian sarcoma virus has also been reported (3).

The induction of lymphoid leukemias by both the proto-oncogene-encoding virus and the 3′ deletion mutant was an unexpected phenotype. We speculate that an important common feature of both viruses is that each expresses a low biological level of p21 protein (compared with wild-type Ha-MuSV). The 3′ deletion mutant virus expresses low levels of authentic viral p21 protein, while the proto-oncogene Ha-MuSV variant expresses high levels of a p21 protein which is less active. Perhaps fibroblasts and splenic erythrocytic precursors require high levels of biologically active p21, leading to pathology with short latency in these target tissues. Under conditions of more modest increases in *ras* activity, the lymphoid tissue may be proportionally more sensitive. Because of its long latency, lymphoid leukemia may not be seen in animals infected with the wild-type Ha-MuSV, since they die rapidly after developing aggressive erythroleukemia and fibrosarcomas. The derivation of the Ha-MuSV LTR from Mo-MuLV, which also induces thymic lymphomas, may also contribute to this susceptibility to thymic disease.

Our studies also indicate that substitution of 60 nt of 5′ noncoding v-*ras*^{H} sequences derived from noncoding c-*ras*^{H} exon −1 for a similar number of nucleotides located just upstream from the first coding exon of c-*ras*^{H} led to a significant increase in transforming activity in vitro and oncogenicity in vivo. Similar increased biological activity was seen for either proto-oncogene- or oncogene-coding sequences. These results may appear to be at variance with those reported by Cichuteck and Duesberg (7). These authors based their constructions on a preliminary oral report of our results and did not make isogenic comparisons. They reported similar transforming activities with wild-type Ha-MuSV and a construction that was virtually identical to pCO32-H″ (which contains the proto-oncogene), perhaps because their transfection assays were less efficient and were made in the presence of helper virus. Our comparisons clearly demonstrate that the biological activity of the proto-oncogene in vitro is lower by more than an order of magnitude than that of wild-type Ha-MuSV and that addition of the 5′ noncoding rat c-*ras*^{H} sequences to virus carrying the two missense mutations further increases their biological activ-

ity. Furthermore, the ability of the 5′ noncoding rat c-*ras*^{H} sequences does not appear to be characteristic of all 5′ noncoding *ras* sequences. Substituting the 5′ noncoding sequences of human c-*ras*^{H} for the same area in v-*ras*^{H} fails to increase the transforming activity of Ha-MuSV encoding either the proto-oncogene or a *ras*^{H} gene with a codon 12 missense mutation (P. E. Tambourin and D. R. Lowy, unpublished data).

It should be noted that our results support the biological relevance of the NIH 3T3 cell transformation assay, which has been questioned by some investigators (10, 19). NIH 3T3 cells are an established, aneuploid line, and results obtained with NIH 3T3 cells have usually been "validated" only by studying the tumorigenic potential of NIH 3T3 cells transformed with these genes. In this report, we have made a more stringent test of the in vitro assay by comparing the transforming activity of variant Ha-MuSV viruses on NIH 3T3 cells with the pathogenic activity of these viruses in vivo. We found a striking correlation between these two independent assays. Two variants which carried very different lesions (pCO23-W and pCO26-C) were found to have low transforming activity on NIH 3T3 cells, to be less pathogenic in vivo, and to induce tumors that differed significantly from those induced by wild-type Ha-MuSV. Conversely, relatively small increases in transforming activity in vitro were correlated with shortened survival time of animals inoculated in vivo (compare, for example, pCO24-I′ and pCO25-J with pCO20-A and pCO22-E).

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LITERATURE CITED

1. **Barbacid, M.** 1987. *ras* genes. Annu. Rev. Biochem. **56**:779–827.
2. **Berns, A. J. M., M. H. T. Lai, R. A. Bosselman, M. A. McKennett, L. T. Bacheler, H. Fan, E. C. Robanus Maandag, H. van der Putten, and I. M. Verma.** 1980. Molecular cloning of unintegrated and a portion of integrated Moloney murine leukemia viral DNA in bacteriophage lambda. J. Virol. **36**:254–263.
3. **Carlberg, K., T. A. Ryden, and K. Beemon.** 1988. Localization and footprinting of an enhancer within the avian sarcoma virus *gag* gene. J. Virol. **62**:1617–1624.
4. **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.
5. **Chang, E. H., P. L. Morgan, E. J. Lee, K. F. Pirollo, E. A. White, and D. H. Patrick.** 1985. Pathogenicity of retroviruses containing either the normal human c-Ha-*ras*1 gene or its mutated form derived from the bladder carcinoma EJ/T24 cell line. J. Exp. Pathol. **2**:177–189.
6. **Chattopadhyay, S. K., A. I. Oliff, D. L. Linemeyer, M. R. Lander, and D. R. Lowy.** 1981. Genomes of murine leukemia viruses isolated from wild mice. J. Virol. **39**:777–791.
7. **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.
8. **Coffin, J.** 1982. Endogenous viruses, p. 1109–1203. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Damante, G., S. Filetti, and B. Rapoport.** 1987. Nucleotide sequence and characterization of the 5′ flanking region of the rat Ha-*ras* proto-ocogene. Proc. Natl. Acad. Sci. USA **84**:774–778.
10. **Duesberg, P. H.** 1987. Cancer genes: rare recombinants instead of activated oncogenes (a review). Proc. Natl. Acad. Sci. USA **84**:2117–2124.
11. **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.
12. **Harvey, J. J.** 1964. An unidentified virus which causes the rapid production of tumours in mice. Nature (London) **204**:1104–1105.
13. **Hawley-Nelson, P., E. J. Androphy, D. R. Lowy, and J. T. Schiller.** 1988. The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. EMBO J. **7**:525–531.
14. **Kauffman, R. S., and B. N. Fields.** 1986. Pathogenesis of viral infections, p. 153–167. *In* B. N. Fields and D. M. Knipe (ed.), Fundamental virology. Raven Press, New York.
15. **Lacal, J. C., E. Santos, V. Notario, M. Barbacid, S. Yamazaki, H.-F. Kung, C. Seamans, S. McAndrew, and R. Crowl.** 1984. Expression of normal and transforming H-*ras* genes in *Escherichia coli* and purification of their encoded p21 proteins. Proc. Natl. Acad. Sci. USA **81**:5305–5309.
16. **Lacal, J. C., S. K. Srivastava, P. S. Anderson, and S. A. Aaronson.** 1986. *ras* p21 proteins with high or low GTPase activity can efficiently transform NIH 3T3 cells. Cell **44**:609–617.
17. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
18. **Li, Y., E. Golemis, J. W. Hartley, and N. Hopkins.** 1987. Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. J. Virol. **61**:693–700.
19. **Littlefield, J. W.** 1982. NIH 3T3 cell line. Science **218**:214–216.
20. **Lowy, D. R., and B. M. Willumsen.** 1986. The *ras* gene family. Cancer Surveys **5**:275–289.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Reddy, E. P., D. Lipman, P. R. Andersen, 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.
23. **Roop, D. R., D. R. Lowy, P. E. Tambourin, J. Strickland, J. R. Harper, M. Balaschak, E. F. Spangler, and S. H. Yuspa.** 1986. An activated Harvey *ras* oncogene produces benign tumours on mouse epidermal tissue. Nature (London) **323**:822–824.
24. **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.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
26. **Schiff, R. D., and A. Oliff.** 1986. The pathophysiology of murine retrovirus-induced leukemias. Crit. Rev. Oncol. Hematol. **5**: 257–323.
27. **Seeburg, P. H., W. W. Colby, D. J. Capon, D. V. Goeddel, and A. D. Levinson.** 1984. Biological properties of human c-Ha-*ras*1 genes mutated at codon 12. Nature (London) **312**:71–75.
28. **Shih, T. Y., and M. O. Weeks.** 1984. Oncogenes and cancer: the p21 *ras* genes. Cancer Invest. **2**:109–123.
29. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1985. Moloney murine leukemia virus (Mo-MLV) genome, p. 766–782. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses: molecular biology of tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Soeda, E., and S. Yasuda.** 1985. Harvey murine sarcoma virus (Ha-MSV) genome, p. 928–939. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses: molecular biology of tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Southern, P. J., and P. Berg.** 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.

32. **Tsuchida, M., E. Ohtsubo, and T. Ryder.** 1982. Nucleotide sequence of the oncogene encoding the p21 transforming protein of Kirsten murine sarcoma virus. Science **217**:937–939.
33. **Velu, T. J., L. Beguinot, W. C. Vass, M. C. Willingham, G. T. Merlino, I. Pastan, and D. R. Lowy.** 1987. Epidermal growth factor-dependent transformation by a human EGF receptor proto-oncogene. Science **238**:1408–1410.
34. **Willumsen, B. M., A. Christensen, N. L. Hubbert, A. G. Papageorge, and D. R. Lowy.** 1984. The p21 *ras* C-terminus is required for transformation and membrane association. Nature (London) **310**:583–586.
35. **Willumsen, B. M., A. G. Papageorge, N. Hubbert, E. Bekesi, H.-F. Kung, and D. R. Lowy.** 1985. Transforming p21 *ras* protein: flexibility in the major variable region linking the catalytic and membrane-anchoring domains. EMBO J. **4**:2893–2896.