Activation of the c-H-ras Proto-Oncogene by Retrovirus Insertion and Chromosomal Rearrangement in a Moloney Leukemia Virus-Induced T-Cell Leukemia

JAMES N. IHLE,^{1,*} BRIAN SMITH-WHITE,¹ BRAD SISSON,¹ DIANA PARKER,¹ DONALD G. BLAIR,² ALAN SCHULTZ,³ CHRISTINE KOZAK,⁴ R. DWAYNE LUNSFORD,^{1,‡} DAVID ASKEW,^{1,†} YACOB WEINSTEIN,^{1,§} AND ROBERT J. ISFORT^{1,†}

Molecular Mechanisms of Carcinogenesis Laboratory¹ and Laboratory of Molecular Virology and Carcinogenesis,³ BRI-Basic Research Program, and Laboratory of Molecular Oncology, National Cancer Institute-Frederick Cancer Research Facility,² Frederick, Maryland 21701, and Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892⁴

Received 1 February 1989/Accepted 31 March 1989

A rearrangement of the c-H-ras locus was detected in a T-cell line (DA-2) established from a Moloney leukemia virus-induced tumor. This rearrangement was associated with the high-level expression of H-ras RNA and the H-ras gene product, p21. DNA from DA-2 cells transformed fibroblasts in DNA transfection experiments, and the transformed fibroblasts contained the rearranged H-ras locus. The rearrangement involved one allele and was present in tissue from the primary tumor from which the cell line was isolated. Cloning and sequencing of the rearranged allele and comparison with the normal allele demonstrated that the rearrangement was complex and probably resulted from the integration of a retrovirus in the H-ras locus between a 5' noncoding exon and the first coding exon and a subsequent homologous recombination between this provirus and another newly acquired provirus also located on chromosome 7. These events resulted in the translocation of the coding exons of the H-ras locus away from the 5' noncoding exon region to a new genomic site on chromosome 7. Sequencing of the coding regions of the gene failed to detect mutations in the 12th, 13th, 59th, or 61st codons. The possible reasons for the complexity of the rearrangement and the significance of the activation of the H-ras locus to T-cell transformation are discussed.

Retroviral integrations are commonly associated with the activation of cellular proto-oncogenes in tumors induced by replication-competent, nontransforming retroviruses (6, 44). Insertional activation of the transforming potential of a cellular gene may result from one of several mechanisms. In some cases, retroviral activation is due to a promoter insertion mechanism and increased expression occurs from a viral 3' long terminal repeat (LTR) or a 5' LTR. In the latter case, transcription can continue through the 3' LTR into cellular sequences or splice out of the virus to cellular sequences. Alternatively, viral insertions can cause deregulated expression of a gene by an enhancer mechanism in which gene expression occurs from the normal promoter under the influence of viral enhancer sequences. Finally, retroviral insertions can result in the expression of altered gene products.

To study the cellular genes involved in the transformation of interleukin-3 (IL-3)-dependent hematopoietic progenitor cells, we have isolated a series of cell lines from Moloney leukemia virus (MoLV)-induced tumors (19) or from Cas-BrM murine leukemia virus-induced tumors (17). The majority of these cell lines are IL-3 dependent for growth and have phenotypic properties of early hematopoietic progenitors. In

addition, IL-3-independent T- and B-lineage cell lines were obtained. To identify potential transforming genes, we have screened these cell lines for rearrangements of known proto-oncogenes and for common sites of viral integrations. These studies have identified rearrangements of the c-myc locus (45, 46) and the activation of a novel zinc finger protein (29) in several of the myeloid-lineage, IL-3-dependent cells. During the course of these experiments, Southern blot analysis with an H-ras-specific probe detected a novel restriction fragment in one of the T-cell lines. Because of the paucity of examples of retroviral activation of the H-ras locus, the rearrangement was further characterized. The results demonstrate that the rearrangement was associated with the high-level expression of the H-ras locus. The rearrangement was due to a complex mechanism which involved viral integration and a recombination event resulting in the translocation of the coding exons of the H-ras locus to a new site on chromosome 7.

MATERIALS AND METHODS

Materials. The BS-9 v-H-ras probe was kindly provided by E. Scolnick (Merck, Sharp & Dohme, West Point, Pa.). The monoclonal antibody (YA6-172) for c-H-ras p21 was kindly provided by Thomas Shih (National Cancer Institute, Bethesda, Md.).

Cell lines. The conditions under which the DA-2 cell line was derived from an MoLV-induced lymphoma have been described previously (18). The RL-12 cell line was derived from a radiation-induced thymic lymphoma cell line from C57BL/Ka mice (6). For routine maintenance, cell lines were grown in RPMI 1640 containing 10% fetal bovine serum.

DNA cloning. The 1T11 clone was obtained by ligating

* Corresponding author.

† Present address: Department of Biochemistry, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38101.

‡ Present address: Laboratory of Cellular and Developmental Biology, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD 20892.

§ Present address: Faculty of Health Sciences, Unit of Microbiology and Immunology, Ben Gurion University of the Negev, Beer Sheva, Israel.

size-selected *Eco*RI-digested DNA to *Eco*RI-digested EMBL 3 DNA, and the DNA was packaged with commercial packaging reactions (Stratagene). Phage plaques (10^6) were screened with the BS-9 probe, and a single positive plaque was detected. The D93 clone was obtained by ligating size-selected *Bam*HI-digested DA-2 DNA with *Bam*HI-digested EMBL 3 DNA, and the DNA was packaged with commercial packaging reactions. Phage plaques (10^6) were screened with the BS-9 probe; while multiple positive clones were detected, only six were characterized. All six had comparable restriction maps, and one clone (D93) was used for further characterization. The DA1ras clone, containing the normal murine c-H-ras locus, was cloned from an EMBL 3 library made with 15- to 20-kilobase (kb), size-selected, *Sau*3A-digested DNA from the DA-1 cell line (19). Size-selected DNAs were prepared by electroelution of DNA from 0.8% agarose gels and purification of the DNA on NACS columns (Bethesda Research Laboratories). Probes for screening the libraries were labeled by random primer synthesis in the presence of [32 P]dCTP. Phage DNA was prepared and restriction maps were developed by standard procedures (39).

Southern and Northern (RNA blot) hybridizations. Cellular DNA was prepared by standard procedures and digested to completion with appropriate restriction enzymes (27). The DNA was electrophoresed in 0.8% agarose gels, and the separated fragments were transferred to nitrocellulose filters as described (39). The filters were hybridized with probes prepared as described above. Total cellular RNA was extracted by the guanidinium isothiocyanate method (5) and was purified by centrifugation through cesium chloride. Poly(A)-containing RNA was isolated by retention on oligo(dT) columns (Collaborative Research). For Northern analysis, 10 μ g of RNA was electrophoresed following denaturation with glyoxal and blotted onto nitrocellulose (42).

DNA sequencing. Appropriate restriction fragments for sequencing were subcloned either into pUC vectors for sequencing by the Maxam and Gilbert procedure (28) or into M13 or Bluescript (Stratagene) for sequencing by the chain termination method (37).

Immunoprecipitations. Immunoprecipitation analysis was performed as has been described previously, using anti-p21 antisera (38, 43). Briefly, 10^7 cells were metabolically labeled with [35 S]methionine, concentrated by centrifugation, lysed in lysis buffer, and precleared with nonimmune sera, and the p21 protein was specifically precipitated with anti-p21 antiserum.

Transformation assays. Transfection analysis was performed according to previously published procedures (1). Briefly, 100 μ g of cellular DNA and 10 μ g of pSV2neo plasmid DNA were transfected into NIH 3T3 cells by the calcium phosphate procedure. Neomycin-resistant colonies were selected in the presence of 400 μ g of G418 per ml. Pools of G418-resistant colonies were either plated for foci formation or used for injection into athymic mice. For each injection, 10^6 G418-resistant cells were used in a saline carrier and tumor formation was followed as indicated.

RESULTS

The DA-2 cell line was isolated from a tumor induced in a BALB/c mouse which had been inoculated at birth with an animal-passaged stock of MoLV. Initially the cells were IL-3 dependent for growth in vitro; however, after approximately 2 months, cell growth became independent of growth fac-

FIG. 1. Rearrangement of the H-ras locus in DA-2 cells. Various DNAs (10 μ g) were digested with *Eco*RI, and restriction fragments were separated by electrophoresis on 0.8% agarose gels. The DNAs were transferred to nitrocellulose, and the blots were hybridized with the BS-9 probe derived from Harvey sarcoma virus (10). The samples included DNAs from cell lines isolated from MoLV-induced tumors (DA-1, DA-2, DA-25), the primary tumors from which the DA-2 cell line was derived (spleen, thymus), clones of fibroblasts transformed with DNA from the DA-2 cell line (1° focus, 2° focus), and the nontransformed parental NIH 3T3 cells. It should be noted that the BS-9 probe contains only 24 bp of the 5' H-ras noncoding exon and therefore does not detect the 5' portion of the rearrangement on Southern blots.

tors. The DA-2 cells express the lymphoid markers LFA-1, Ly-5, and Thy-1.2 but do not express Ly-1 or Ly-2 (data not shown). The myeloid lineage markers RB6-8C5, Mac-1, Mac-2, and Mac-3 are not expressed. DA-2 cells contain rearrangements of both T-cell receptor β -chain alleles (data not shown). Taken together, the phenotypic characteristics indicate that the DA-2 cells are of the T-cell lineage.

To determine the potential role of rearrangements of the c-H-ras locus in MoLV-induced tumors, DNAs from a variety of tumors and cell lines were examined by Southern blot analysis. *Eco*RI-digested DA-2 DNA contained a unique 18-kb restriction fragment when the blots were probed with the BS-9, murine v-H-ras probe (10) (Fig. 1). No rearrangements were detected with DNA from DA-1 or DA-25 cells (Fig. 1) or with DNAs from approximately 40 additional MoLV-induced tumors or cell lines (data not shown). With DA-2 DNA, the intensity of the signal from the unique fragment was comparable to that of the normal allele, indicating that the rearrangement involved one of the two cellular alleles. The unique fragment was also detected in thymus and spleen DNA from the primary tumor (1° thymus, 1° spleen), although the intensity was less than that of the normal allele (Fig. 1), suggesting that the cells with the rearrangement constituted only a fraction of the tumor tissue. Whether this is due to the rearrangement being present in a subpopulation of transformed cells or is due to infiltration of the tumor by normal cells could not be determined.

DNA from DA-2 cells was examined for the ability to transform NIH 3T3 cells in transfection assays in vitro and to induce tumors in an in vitro/in vivo transformation assay (1). DA-2 DNA caused morphological transformation of NIH 3T3 cells (Table 1). In two separate experiments the efficiency of transformation was five transformed foci per 100 μ g of DA-2 DNA. In two additional experiments in which 100 μ g of DA-2 was cotransfected with pSV2neo, the

TABLE 1. Transforming activity of DA-2 DNA^a

DNA	In vitro assays: no. of transformed foci		In vitro/in vivo assays: tumors/total (latency, wks)
	Per 100 µg of DNA	Per 10 ⁶ G418-resistant	
DA-2	5, 5	14, 2	5/5 (4-8); 1/6 (12); 4/5 (6-7); 2/4 (9)
86-2	NT	27	3/3 (3)
C605	NT	NT	2/3 (5)
NIH 3T3	0, 0, 0	NT	0/7; 1/5 (9); 0/6
DA-2 1° focus	NT	NT	5/6 (5-6)
DA-2 1° tumor	NT	NT	6/6 (5-7)

^a Transfection assays were performed according to previously published procedures (1). In standard in vitro assays, 100 µg of DNA was transfected onto NIH 3T3 cells and the number of transformed foci per 100 µg of DNA was determined. Alternatively, 100 µg of DNA was transfected in the presence of 10 µg of DNA from the plasmid pSV2neo. G418-resistant cells were selected, and the number of transformed foci per 10⁶ G418-resistant cells was determined. In the in vitro/in vivo assays, NIH 3T3 cells were transfected with 100 µg of DNA in the presence of 10 µg of pSV2neo DNA and G418-resistant cells were selected. G418-resistant cells (10⁶) were injected into individual mice, and the frequency of tumors and the time of latency were determined. DNAs from a mouse tumor (86-2) containing an activated *H-ras* gene and a cell line carrying a transfected *v-K-ras* gene are included as positive controls. NT, Not tested.

efficiency of transformation was 14 and 2 foci per 10⁶ G418-resistant cells. For comparison, 100 µg of DNA from a mouse tumor (86-2), containing an activated *H-ras* gene, transformed with an efficiency of 27 foci per 10⁶ G418-resistant cells per 100 µg of DNA. No transformed foci were seen with DNA from NIH 3T3 cells. When NIH 3T3 cells were transfected with 100 µg of DA-2 DNA in the presence of pSV2neo and selected for G418 resistance, and 10⁶ cells were inoculated into individual mice, tumors were observed at the frequencies of 5/5, 1/6, 4/5, and 2/4 in individual experiments. In contrast, DNA from NIH 3T3 cells only resulted in a single tumor in one mouse in one of the three experiments (Table 1), and DNAs from two other MoLV-induced tumors were not transforming in this assay (data not shown). Lastly, DNA from a primary in vitro-transformed focus (1° focus) or from a tumor from the in vitro/in vivo assay (1° tumor) had transforming activity in the in vitro/in vivo assay.

To determine whether the transforming activity of DA-2 DNA was due to the rearranged *H-ras* gene, DNAs from in vitro-derived transformed foci or from tumors obtained in the in vitro/in vivo assay were examined by Southern blot analysis. Results (Fig. 1) demonstrated that DNA from an in vitro focus (1° focus) and from a secondary focus (2° focus) obtained from the DNA from the primary focus contained an altered *H-ras* hybridizing *Eco*RI fragment. In this particular case, the restriction fragment size was smaller than that seen in DA-2 DNA, suggesting that additional rearrangements had occurred during transfection. Analysis of DNAs from two additional foci and four tumors, however, contained the characteristic rearranged *Eco*RI fragment seen in DA-2 cells (data not shown).

The expression of the *c-H-ras* gene in DA-2 cells was examined by Northern analysis and by immunoprecipitation. DA-2 cells expressed high levels of a 1.1-kb *H-ras* transcript as compared with a T-cell line (RL-12) which had no detectable rearrangement of the *c-H-ras* locus (Fig. 2). Immunoprecipitation results, utilizing a *H-ras*-specific monoclonal antibody (13), are shown (Fig. 2B). DA-2 cells had high levels of p21 (Fig. 2B) which were comparable to the levels found in Harvey sarcoma virus-transformed fibro-

FIG. 2. Expression of *H-ras* RNA and levels of p21 in DA-2 cells. (A) Poly(A)⁺ RNA was isolated as described in Materials and Methods from DA-2 cells and a T-cell line isolated from a radiation-induced thymic lymphoma in a C57BL/6 mouse. A 10-µg sample of RNA and separated on 1% agarose gels following denaturation with glyoxal and was subsequently transferred to nitrocellulose. The blots were hybridized with the BS-9 probe as described for Fig. 1. (B) The DA-2 cell line and other cell lines isolated from MoLV-induced tumors were labeled with [³⁵S]methionine, collected, and lysed as previously described, and the extracts were immunoprecipitated with a monoclonal antibody which specifically detects *H-ras* p21 (13).

blasts (data not shown). In contrast, other murine leukemia virus-induced tumor cell lines contained no detectable *H-ras* p21. Taken together, these results indicate that the rearrangement was associated with the high-level expression of the *c-H-ras* locus.

To further characterize the rearrangement in DA-2 cells, the rearranged *c-H-ras* locus and the normal murine *c-H-ras* locus were cloned and compared. Initially the BS-9 probe was used to screen a genomic library made with size-selected, *Eco*RI-digested DA-2 DNA, and a single clone (1T11) was isolated which contained the 5' region of the *c-H-ras* gene as described below. Since the 3' region of the *c-H-ras* gene was not obtained from this library, the BS-9 probe was used to screen a genomic library made with size-selected, *Bam*HI-digested DA-2 DNA. From this library several identical clones were isolated, and one (D93) was further characterized. The normal allele was cloned from a *Sau*3A partial library.

The structures of the clones were deduced by restriction mapping and partial sequencing (Fig. 3). From the restriction map, the 1T11 clone (Fig. 3, bottom) contained a 5' LTR and a complete provirus up to an *Eco*RI site in the *env* gene region. The position of this *Eco*RI site is consistent with the presence of a mink cell focus-forming provirus (2, 32), and sequencing of the LTR demonstrated its origin from MoLV (data not shown). The restriction map of the cellular sequences 5' of the provirus was not that expected for the coding regions of the *H-ras* locus, and therefore the region hybridizing with the BS-9 probe was subcloned and sequenced. Approximately 522 base pairs (bp) 5' of the viral LTR (Fig. 4, underlined sequence), there was a region which contained approximately 130 bp of sequence found in *H-ras*, 24 bp of which is found in the BS-9 probe. The region also had striking homology to sequences present in the 5' region of the human *H-ras* locus, which has been shown to contain a 5' noncoding exon (exon -1) of the human *c-H-ras* locus

FIG. 3. Structure of the 1T11 and D93 clones containing the rearranged H-ras locus from DA-2 cells. The 1T11 clone (bottom) was isolated from a genomic library of *Eco*RI-restricted, size-selected DNA from DA-2 cells in EMBL 3. The D93 clone (top) was isolated from a genomic library of *Bam*HI-restricted, size-selected DNA from DA-2 cells in EMBL 3. The positions of the viral sequences and the locations of the H-ras exons were determined by restriction mapping and partial sequencing by standard techniques. The positions of the sites for *Eco*RI (R), *Bam*HI (B), *Hind*III (H), *Xba*I (Xb), *Xho*I (X), *Sma*I (Sm), and *Sac*I (S) are shown. For comparison, the structure of a clone containing the normal H-ras locus which was isolated from a *Sau*3A partial genomic library made in the EMBL 3 vector is shown (center). The underlined areas indicate regions which have restriction sites, or sequences, which are characteristic of MoLV proviruses. The underlined unique cellular sequences are sequences which were translocated next to the H-ras coding exons as described in the text.

(18, 20, 21). These results suggested that an MoLV-mink cell focus-forming virus had integrated between the 5' noncoding exon and the first coding exon of the H-ras locus.

To characterize the other side of the rearrangement, the D93 clone was isolated from a *Bam*HI library. Restriction mapping of this clone demonstrated the presence of a deleted provirus of 3.5 kb containing an *Eco*RI site (Fig. 3, top). The sequence around the *Eco*RI site (data not shown) indicated that the provirus was derived from an MoLV-mink cell focus-forming virus. The restriction map of the sequence 3' of the provirus was that expected of the H-ras locus. Partial sequencing of this region identified three of the four coding exons of the H-ras locus. The 3' *Bam*HI site was located in intron sequences between the third and the fourth exon (Fig. 3), and consequently the clone does not contain the complete coding region of the gene. Unexpectedly, the region 5' of the provirus in the D93 clone was 9.5 kb of non-c-H-ras sequence. The structures of the 1T11 and D93 clones indicated that the rearrangement in DA-2 cells involved more than a simple proviral insertion in the c-H-ras locus.

To confirm the structure of the rearranged allele deduced from the restriction maps of the 1T11 and D93 clones and to demonstrate that the sequences found 5' to the provirus in the 1T11 clone are normally immediately 5' to the sequences found 3' of the provirus in the D93 clone, Southern blot analysis was done. For these experiments, cellular probes from the 1T11 and D93 clones were used. Figure 5A shows the results obtained with a *Hind*III-*Bam*HI probe from the D93 clone which contains three of the four coding exons of the H-ras locus. This probe detected a *Bam*HI fragment of 3.5 kb which is found in normal DNA (restriction fragments found in normal DNA are indicated by dots in Fig. 5) as well as a fragment of the size of the *Bam*HI insert of the D93 clone. This probe also detected *Xba*I and *Kpn*I fragments of approximately 9.5 and 6.6 kb, respectively, which are found in normal DNA. In addition, the D93 probe detected unique fragments of the sizes predicted from the restriction map of the clone. Figure 5B depicts the results obtained with an *Xho*-*Xho* probe which is immediately 5' of the viral LTR in the 1T11 clone. Importantly, this probe detected *Bam*HI, *Xba*I, and *Kpn*I fragments in normal DNA and in DA-2 DNA

(indicated by dots) which comigrated with the fragments detected with the cellular probe from the 1T11 clone. These results demonstrate that the sequences 5' of provirus in the 1T11 clone lie within the same restriction fragments as the sequences immediately 3' of the provirus in the D93 clone. Furthermore, the results indicate that with the restriction enzymes used there is no apparent rearrangement of the second H-ras locus in DA-2 cells. Lastly, the unique restriction fragments detected with the D93 probe are consistent with the restriction map of this clone. Taken together, these results demonstrate that the rearrangement in DA-2 cells involved one allele of the H-ras gene and that the structure of the phage clones reflects the genomic structure.

The structures of the clones suggested the possibility that the rearrangement might have first involved a viral integration in the intron between exon -1 and the first coding exon, followed by recombination with another provirus, resulting in the translocation of the coding region of the H-ras locus to a new chromosomal site. If this model is correct, in the normal H-ras allele the sequences 5' of the LTR in the 1T11 clone should be continuous with the sequences 3' of the LTR in the D93 clone, as suggested by the Southern blot analysis data in Fig. 5. Moreover, there should be a four-base repeat which was generated during the initial integration event (9). To examine this, the region of the normal allele in which the rearrangements occurred was sequenced and this sequence was compared with the sequences at the proviral integration sites. The sequences from the 1T11 clone and the D93 clone allowed the identification of a unique integration site (Fig. 6). In addition, at the 3' LTR in D93, there are four extra bases between the site of the integration and the end of the LTR. However, the origin of these four bases is not clear since they are not a direct repeat of the sequences at the 5' LTR of the 1T11 clone, nor are they a repeat of the sequences at the 5' LTR of the D93 clone.

The above results indicated that the rearrangement in DA-2 cells might have arisen by recombination between two proviruses, resulting in the translocation of the coding region of the H-ras locus to a new chromosomal location. It was therefore of interest to chromosomally map the 5' cellular sequences found in the D93 clone. Using DNA from a panel

DA-2 ras	AAGCAGGTC TCGGTCTGTC TCTTCTGCAC CTAGGCACTA GACATCACTG TTGAGTGCAG
DA-2 ras	GACCCAAGTT CCGGCACTGC CTTCCCCGCG GCTCTAGGAA GCCCCGCCCA GCTTCGGCTT
DA-2 ras	CTGACGGATG GGTGTCTCAG CCAACCACAA CAGGCGCGGT GCGGAGGAGT CTGACCAATC
DA-2 ras	ACGGGCGCGC TCGAGTTGAC GGACGGGCGT GGGCGGAGCG <u>GGGCGGGCGG</u> AGCGCGGGCC
Hu ras	::G: A::::A::G :C::::G::: ::::::::::: T::::A:::::
DA-2 ras	CGGCCAAGCC CCCGCCCTCC GCTCCCGGCG CCCGCAGCCC GAGCCGCACC CGCCGCGGAC
Hu ras	::C::G::T: T:::::C::: :TG:::T::: ::::::::::: A::::: ::::::::::: :::::::::::
v ras	: :::::::::::G::::::::::: ::::::::::: :::::::::::
DA-2 ras	GGAGCTCATG CGCGGGCCCA GCCG-GCGCC C--GTCCGCG CCCCGGCCCT GCCCGGGCCC
Hu ras	:::::C::::: ::::::::::G:G: A:::C::::: :CC:C::C::: ::::GC:::G :::T:::::
v ras	:::::C::::: ::::::::::: :T::::::::::: ::::::::::: ::::::::::: :::::::::::
DA-2 ras	CGGCCCCGCG CCCGGGGGCA GTCGCGCCAG -CAAGCGGTG AGTGGCGGCA CGGGGTGGGG
Hu ras	:::::T::: ::::::::::: :::::::::::T: TG:-:-----: ::::::::::: G:::
v ras	::::::::::: ::::::::::: ::::::::::: :::::::::::
DA-2 ras	CGCGGGGCGC AGGTGATCGG TGCTTCCTTG CTCTCTCGG CCGGCGAGCG TGCCTAGCGT
DA-2 ras	CCGGCTGCCC GCCTCTGCGG CCGGACTCGC TCTGCCGTGTG GCGGTCTCTC CAGCGAGGCG
DA-2 ras	CCGCCCCGGC CTCCGGGGAC TCTGCGCCCC GCGGCCATCA CGCTCGGGCC TCGGGGCACA
DA-2 ras	CAAGGACACC CGCGTTGGGC CCCAACCAAG GCCTTCTGCT CCCAGCCATC CCTCGCGTTC
DA-2 ras	CTGTAGTCAC CTGCTTCCCA CACCCTCAGC CGTTTTTCCC CCCTCTGTGC AAAGCTCCCT
DA-2 ras	AGCCCTTGT GGAAGGTTTG TCTTAGTGA GGGCAGGTC CTTCTCCAGC CACCCTCCCC
DA-2 ras	CCGCTGCCCG GCCCGGCCTA GCATGCAGTG GCGCTCAATA ATAGTTGTAG GTTGCACCCA
DA-2 ras	CATGCCGGCG GGCCAGAGCC GGTGTAGGC TGGCATT-GC CTGCCTAGGC ACGCCGAGGC
DA-2 ras	CGCCGTGGCA GGACCTGTTT TTGGGGGGTG GCCTTGAAG <u>ACCCACCCG</u> <u>TAGGTTGGC</u>
DA-2 ras	<u>CGAGCTAGAC</u>

FIG. 4. Comparison of the murine and human sequences of the 5' noncoding exon of the H-ras locus. The *Bam*HI-*Xba*I fragment, containing the BS-9 hybridizing region, of the 1T11 clone and the normal c-H-ras clone were subcloned into pUC12 vector and sequenced by the procedures of Maxam and Gilbert (28) or by chain termination methods (37). For comparison, the sequence of the comparable region of the human H-ras locus, taken from reference 3, is shown. The v-H-ras sequence corresponding to the exon -1 region has been described previously. The viral integration site in the 1T11 clone is located 522 bp 3' of the end of the exon, and the viral LTR sequences are underlined.

of hamster-mouse somatic cell hybrids and the *Xba*I-*Xba*I probe, the 5' sequences in D93 were localized to chromosome 7, the same chromosome that contains the c-H-ras locus (25, 36) (Table 2). These data indicate that recombination involved an intrachromosomal translocation event catalyzed by two proviruses integrated at different locations on chromosome 7.

The results indicated that activation of the H-ras locus in DA-2 cells involved retroviral insertion and a genomic rearrangement. Although previous studies have demonstrated that overexpression of the normal H-ras gene from an LTR promoter can cause transformation, it was still important to determine whether the H-ras in DA-2 cells also contained any of the mutational events (codons 12, 59, or 61) which are normally involved in the activation of the transforming activity of ras genes (11, 35, 40, 41, 48). We therefore sequenced these regions of the rearranged locus from DA-2 cells. The sequences around these codons were identical to the sequences reported for the normal human or rat c-H-ras locus (data not shown).

DISCUSSION

The results presented here demonstrate a complex retroviral insertional activation of the c-H-ras gene in a murine

FIG. 5. Characterization of the rearrangement in DA-2 cells with probes from the 1T11 and D93 clones. DA-2 DNA (10 µg) was digested with *Bam*HI, *Xba*I, and *Kpn*I, electrophoresed on 0.8% agarose gels, and transferred to nitrocellulose. The blots were then hybridized with either an *Xho*-*Xho* fragment derived from the 1T11 clone or a *Hind*III-*Bam*HI fragment isolated from the D93 clone (A). The positions of the restriction fragments seen with these probes in normal DNA (B) are indicated by the dots.

IT11 5' LTR TGTTTTTTGGGGGTTGCCCTTGAAAGACCCACCCCGT
 :::~::~:
Normal allele TGTTTTTTGGGGGTTGCCCTCAGCCCTCAGGC GCCTCC
 :::~::~:
D93 3' LTR GTCAGCGGGGCTCTTCA**T**TCTCAGCCCTCAGGC GCCTCC

D93 5' LTR TATCAGCTTCAAATGATG**TATT**TGAAGACCCACCCCGT

FIG. 6. Comparison of viral LTR-cellular junction sequences with sequences of the preintegration site. Sequencing of the LTR junctions from IT11 and D93 and the comparable region of the normal allele was accomplished as described in Materials and Methods.

T-cell leukemia cell line. This is one of the few activations of the *H-ras* locus which does not involve mutation of codons 12, 59, or 61. The overexpression of a nonmutated c-*H-ras* gene has been implicated in the transformation of a chicken nephroblastoma cell line (47), although the genomic rearrangements involved in this activation were not characterized. The overexpression of a nonmutated c-*K-ras* gene, due to a retroviral insertion, has been implicated in the transformation of a murine myeloid cell line (14). The ability of viral LTR-mediated overexpression of the normal c-*H-ras* allele to cause transformation has been previously demonstrated in *in vitro* experiments (4, 8).

The characterization of the rearrangement in DA-2 cells resulted in the identification of the murine *H-ras* 5' noncoding exon. An *H-ras* 5' noncoding exon was first identified in the human *H-ras* locus by primer extension analysis (21). In the 1T11 clone there was a 200-bp region which showed extensive homology to the presumptive 5' noncoding exon of the human gene and was located at the same position relative to the first coding exon. By comparing the sequences, a portion of this exon has been transduced into the v-*H-ras* gene in Harvey sarcoma virus as well as into the v-*H-ras* genes of Rasheed virus (33) and BALB/c murine sarcoma

virus (34), although to varying extents. The murine exon -1 contains multiple stop codons in all reading frames and therefore is unlikely to contribute to the H-*ras* gene product. Similar to the human gene, the 5' region of the exon is G+C rich and contains several potential Sp 1 binding sites. One of these binding sites is conserved between the human and murine genes (Fig. 4) and consequently may be important in regulation.

The retroviral activation of the *H-ras* locus in DA-2 cells was complex. Taken together, the results suggest that initially a virus integrated between the 5' noncoding and the first coding exon. Subsequently, this provirus catalyzed a homologous recombination with another provirus on the same chromosome, resulting in the translocation of the coding sequences to a new chromosomal site. The strongest evidence for this model is the ability to define a unique integration site in the normal allele from the sequences at the LTR-cellular junctions in the 5' LTR of the 1T11 clone and the 3' LTR of the provirus in the D93 clone. Because of this, it was surprising that a terminal redundancy could not be identified. The basis for this is not known; however, it is important to note also that no terminal redundancy was detectable in the LTR-cellular junctions in the provirus in the D93 clone.

A similar type of complex rearrangement may be responsible for the activation of the *c-myc* locus in an MoLV-induced thymic lymphoma in rats (26). In this case a recombination occurred between a provirus integrated adjacent to *c-myc* and a provirus elsewhere on the same chromosome. The resulting provirus had additional copies of the LTR enhancer, and this was speculated to increase the level of *c-myc* transcription from this locus.

The basis for the complexity in the rearrangement in DA-2 cells is not known, but presumably it is related to the level of expression that is required for transformation. For this reason, it can be speculated that the initial retroviral integration did not result in high levels of H-*ras* expression. This may be due to the presence of a complete provirus in the initial integrative event which resulted in the inefficient usage of the 3' LTR as a downstream promoter, as suggested in avian leukosis virus activation of the c-*myc* locus (12, 15, 30). Recombination therefore resulted in the positioning of a deleted provirus with a more effective 3' LTR upstream from the H-*ras* locus. Alternatively, the H-*ras* locus might be transcriptionally inactive and recombination was required to move the integration site to a more transcriptionally active region. This possibility is supported by the observation that Northern analysis with probes from the 5' noncoding exon indicated no transcription of this region from either the normal allele or the rearranged allele in DA-2 cells (data not shown).

The significance of the overexpression of the *H-ras* locus to the transformation of the DA-2 cells is not known. A role for the mutational activation of the members of the *ras* gene family in T-cell transformation has been previously demonstrated (16, 31). It is possible that altered *ras* gene expression affects growth regulation, growth factor dependence, or differentiation of the T cells. In previous studies we have demonstrated that Harvey sarcoma virus alters the growth rate of IL-3-dependent cells but has no effect on the growth factor dependence of the cells or on the ability of progenitor cells to terminally differentiate. Whether v-*H-ras* has comparable effects on T cells is currently being examined.

TABLE 2. Chromosomal localization of the *Xba*I-*Xba*I restriction fragment from the D93 clone^a

Mouse chromosome	No. of hybrid clones (positive/chromosome retention)				% Discordance
	+/+	-/-	+/-	-/+	
1	4	1	3	3	54
2	5	1	3	3	50
3	5	1	3	3	50
4	1	3	7	1	67
5	0	4	7	0	64
6	5	1	3	3	50
7	8	4	0	0	0
8	2	2	6	2	67
9	3	3	5	1	50
10	0	4	8	0	66
11	0	4	7	0	64
12	6	1	2	3	42
13	3	2	3	1	44
14	2	4	6	0	50
15	7	0	0	4	36
16	2	1	5	1	67
17	6	1	2	3	42
18	3	1	5	2	64
19	4	2	4	2	50
X	3	1	5	3	67

^aHybrid cell lines were derived from fusion of E36 Chinese hamster cells with cells from BALB/c, NFS, or A/J mice. The chromosomal content of each line was determined by Giemsa-trypsin banding, followed by staining with Hoechst 33258 and by expression of isozyme loci on 13 of the mouse chromosomes (22-25).

ACKNOWLEDGMENTS

Research was sponsored by the National Cancer Institute under Public Health Service contract NO1-CO-74101 with Bionetics Research, Inc. This work was also supported in part by Public Health Service National Cancer Institute Cancer Center Support (CORE) grant P30 CA21765 and by the American Lebanese Syrian Associated Charities (ALSAC).

LITERATURE CITED

- Blair, D. G., C. S. Cooper, M. K. Oskarsson, L. A. Eader, and G. F. Vande Woude. 1982. New method for detecting cellular transforming genes. *Science* **218**:1122-1125.
- Bosselman, R. A., F. van Straaten, C. Van Beveren, I. M. Verma, and M. Vogt. 1982. Analysis of the *env* gene of molecular clones and biologically active Moloney mink cell focus-forming proviral DNA. *J. Virol.* **44**:19-31.
- 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. 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.
- Chirgwin, J., A. Przybyla, R. MacDonald, and W. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
- Clurman, B. E., and W. S. Hayward. 1988. Insertional activation of protooncogenes previously identified as viral oncogenes, p. 55-94. *In* G. Klein (ed.), *Cellular oncogene activation*. Marcel Dekker, Inc., New York.
- Decleve, A., M. Lieberman, J. N. Ihle, P. N. Rosenthal, M. L. Lung, and H. S. Kaplan. 1978. Physicochemical, biological, and serological properties of a leukemogenic virus isolated from cultured RadLV-induced lymphomas of C57BL/Ka mice. *Virology* **90**:23-35.
- 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.
- Dhar, R., W. L. McClements, L. W. Enquist, and G. F. Vande Woude. 1980. Nucleotide sequences of integrated Moloney sarcoma provirus long terminal repeats and their host and viral junctions. *Proc. Natl. Acad. Sci. USA* **77**:3937-3941.
- 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.
- Fasano, O., T. Aldrich, R. Tananoi, E. Taprowsky, M. E. Furth, and M. Wigler. 1984. Analysis of the transforming potential of the human H-*ras* gene by random mutagenesis. *Proc. Natl. Acad. Sci. USA* **81**:4008-4012.
- Fung, Y.-K., A. M. Fadley, L. B. Crittenden, and H.-J. Kung. 1981. On the mechanism of retrovirus-induced avian lymphoid leukosis: deletion and integration of the provirus. *Proc. Natl. Acad. Sci. USA* **78**:3418-3422.
- 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.
- George, D. L., B. Glick, S. Trusko, and N. Freeman. 1986. Enhanced c-Ki-ras expression associated with Friend virus integration in a bone marrow-derived mouse cell line. *Proc. Natl. Acad. Sci. USA* **83**:1651-1655.
- Goodenow, M. M., and W. S. Hayward. 1987. 5' Long terminal repeats of *myc*-associated proviruses appear structurally intact but are functionally impaired in tumors induced by avian leukosis viruses. *J. Virol.* **61**:2489-2498.
- Guerrero, I., P. Calzada, A. Mayer, and A. Pellicer. 1984. A molecular approach to leukemogenesis: mouse lymphomas contain an activated c-*ras* oncogene. *Proc. Natl. Acad. Sci. USA* **81**:202-205.
- Holmes, K. L., E. Palaszynski, T. N. Fredrickson, H. C. Morse 3d., and J. N. Ihle. 1985. Correlation of cell-surface phenotype with the establishment of interleukin 3-dependent cell lines from wild-mouse murine leukemia virus-induced neoplasms. *Proc. Natl. Acad. Sci. USA* **82**:6687-6691.
- Honkawa, H., W. Masahashi, S. Hashimoto, and T. Hashimoto-Gotoh. 1987. Identification of the principal promoter sequence of the c-H-*ras* transforming oncogene: deletion analysis of the 5'-flanking region by focus formation assay. *Mol. Cell. Biol.* **7**:2933-2940.
- Ihle, J. N., A. Rein, and R. Mural. 1984. Immunological and virological mechanisms in retrovirus induced murine leukemogenesis. *Adv. Viral Oncol.* **4**:95-137.
- Ishii, S., J. T. Kadonaga, R. Tjian, J. N. Brady, G. T. Merlino, and I. Pastan. 1986. Binding of the Sp 1 transcription factor by the human Harvey *ras* 1 proto-oncogene promoter. *Science* **232**:1410-1413.
- Ishii, S., G. T. Merlino, and I. Pastan. 1985. Promoter region of the human Harvey *ras* proto-oncogene: similarity to the EGF receptor proto-oncogene promoter. *Science* **230**:1378-1381.
- Kozak, C., E. Nichols, and F. H. Ruddle. 1975. Gene linkage analysis in the mouse by somatic cell hybridization: assignment of adenine phosphoribosyl-transferase to chromosome 8 and alpha-galactosidase of the X chromosome. *Somatic Cell Genet.* **1**:371-382.
- Kozak, C. A., J. B. Lawrence, and F. H. Ruddle. 1977. A sequential staining technique for the chromosomal analysis of the interspecific mouse/hamster and mouse/human somatic cell hybrids. *Exp. Cell. Res.* **105**:109-117.
- Kozak, C. A., and W. P. Rowe. 1979. Genetic mapping of the ecotropic murine leukemia virus-inducing locus of BALB/c mouse to chromosome 5. *Science* **204**:69-71.
- Kozak, C. A., J. F. Sears, and M. D. Hoggan. 1983. Genetic mapping of the mouse oncogenes c-Ha-*ras* 1 and c-*fes* to chromosome 7. *J. Virol.* **47**:217-220.
- Lazo, P. A., and P. N. Tsichlis. 1988. Recombination between two integrated proviruses, one of which was inserted near c-*myc* in a retrovirus-induced rat thymoma: implications for tumor progression. *J. Virol.* **62**:788-794.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499-560.
- Morishita, K., D. S. Parker, M. L. Mucenski, N. A. Jenkins, N. G. Copeland, and J. N. Ihle. 1988. Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. *Cell* **54**:831-840.
- Payne, G. S., S. A. Courtneidge, L. B. Crittenden, A. M. Fadly, J. M. Bishop, and H. E. Varmus. 1981. Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. *Cell* **23**:311-322.
- Pierce, J. H., and S. A. Aaronson. 1982. BALB- and Harvey-murine sarcoma virus transformation of a novel lymphoid progenitor cell. *J. Exp. Med.* **156**:873-887.
- Quint, W., W. Quax, H. van der Putten, and A. Berns. 1981. Characterization of AKR murine leukemia virus sequences in AKR mouse substrains and structure of integrated recombinant genomes in tumor tissues. *J. Virol.* **39**:1-10.
- Rasheed, S., G. L. Norman, and G. Heidecker. 1983. Nucleotide sequence of the Rasheed rat sarcoma virus oncogene: new mutations. *Science* **221**:155-157.
- Reddy, E. P., D. Lipman, P. R. Anderson, S. R. Tronick, and S. A. Aaronson. 1985. Nucleotide sequence analysis of BALB/c murine sarcoma virus transforming gene. *J. Virol.* **53**:984-987.
- 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.
- Sakaguchi, A. Y., P. A. Lalley, B. V. Zabel, R. W. Ellis, E. M. Scolnick, and S. L. Naylor. 1984. Chromosomal assignments of

- four mouse cellular homologs of sarcoma and leukemia virus oncogenes. *Proc. Natl. Acad. Sci. USA* **81**:525-529.
37. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA-sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463-5467.
 38. Schultz, A. M., E. H. Rabin, and S. Oroszlan. 1979. Post-translational modification of Rauscher leukemia virus precursor polyproteins encoded by the *gag* gene. *J. Virol.* **30**:255-266.
 39. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503-517.
 40. Tabin, E. 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. 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.
 42. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* **77**:5201-5205.
 43. Tsuchiya, M., Y. Kaziro, and S. Nagata. 1987. The chromosomal gene structure for murine granulocyte colony-stimulating factor. *Eur. J. Biochem.* **165**:7-12.
 44. Varmus, H. E. 1984. The molecular genetics of cellular oncogenes. *Annu. Rev. Genet.* **18**:553-612.
 45. Weinstein, Y., J. L. Cleveland, D. S. Askew, U. R. Rapp, and J. N. Ihle. 1987. Insertion of truncation of *c-myb* by murine leukemia virus in a myeloid cell line derived from cultures of normal hematopoietic cells. *J. Virol.* **61**:2339-2343.
 46. Weinstein, Y., J. N. Ihle, S. Lavu, and E. P. Reddy. 1986. Truncation of the *c-myb* gene by a retroviral integration in an interleukin-3 dependent myeloid leukemia cell line. *Proc. Natl. Acad. Sci. USA* **83**:5010-5014.
 47. Westaway, D., J. Papkoff, C. Moscovici, and H. E. Varmus. 1986. Identification of a provirally activated *c-Ha-ras* oncogene in an avian nephroblastoma via a novel procedure: cDNA cloning of a chimeric viral-host transcript. *EMBO J.* **5**:301-309.
 48. Yuasa, Y., S. K. Srivastava, C. Y. Dunn, J. S. Rhim, E. P. Reddy, and S. A. Aaronson. 1983. Acquisition of transforming properties by alternative point mutations within *c-bas/has* human proto-oncogene. *Nature (London)* **303**:775-779.