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Chromosomal Localization of Three Human *Ras* Genes by In Situ Molecular Hybridization

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Abstract—*Three human* ras *family protooncogenes, c-Ki*-ras-*1, and c-Ki*-ras-*2, and N*-ras, *have been mapped to chromosome bands 6p11-12, 12p11.1-12.1, and 1p11–13, respectively by in situ molecular hybridization. Certain human cancers display consistent and specific alterations involving chromosomes 1, 6, and 12. The precise chromosomal localization of* ras *genes will permit evaluation of the possible effect of these chromosome changes on the structure and activities of* ras *protooncogenes in human neoplasia.*

INTRODUCTION

The identification of human genes with the ability to transform appropriate cells has been made possible by the application of DNA transfection techniques (1, 2). The vast majority of such transforming genes are related to members of the *ras* family of retroviral transforming genes (3–8). The *ras* gene family consists of c-Ha-*ras*-1 and c-Ha-*ras*-2 (9), the homologs of the Harvey-murine sarcoma virus-transforming gene; c-Ki-*ras*-1 and c-Ki-*ras*-2, the homologs of Kirsten-murine sarcoma virus (9); and N-*ras* for which no viral counterpart has yet been isolated (6). These genes are distantly related to one another based on nucleotide sequence similarities, although the transforming proteins they encode possess highly similar amino acid sequences (10–14).

A large body of evidence has accumulated regarding the malignant activation of these genes in human tumors. Thus, single-point mutation in either of two specific codons confers upon human *ras* oncogenes the ability to transform mouse NIH/3T3 cells (13, 15–20). Furthermore, these mutations are found in the *ras* genes of the tumor cells but not in the normal cells of cancer patients (16, 21, 22). The *ras* protooncogenes can also be activated by increasing their level of expression by in vitro manipulations which provide new regulatory sequences for their transcription (i.e., retroviral LTRs) (23, 24) (A. Gazit, A. Srinivasan, C.Y. Dunn, and S.A. Aaronson, manuscript in preparation).

Because of interest in understanding the interactions of human *ras* protooncogenes with other genetic elements, efforts have been directed to chromosomally localizing these genes. Panels of human–rodent somatic cell hybrids and filter hybridization techniques

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were used to assign c-Ki-*ras*-1, c-Ki-*ras*-2, and N-*ras* to chromosomes 6(pter–13), 12, and 1, respectively (25). In the present study, in situ molecular hybridization has been used to further localize these genes.

MATERIALS AND METHODS

Cell Cultures and Chromosome Preparations. Human blood lymphocyte cultures were initiated by mixing 1.0 ml of whole, heparinized peripheral blood with 9.0 ml of RPMI 1640 medium supplemented with 15% fetal bovine serum (CM) and 0.1 ml phytohemagglutinin (M form) (Gibco Laboratories, Grand Island, New York). Methotrexate (10^{-7} M) (Lederle, Pearl River, New York) was added 72 h later, and incubation continued for an additional 17 h. The cultures were centrifuged (1200 rpm) for 8 min, washed twice with Hanks' balanced salt solution (HBSS), and reincubated for 5 h in CM containing thymidine (10^{-5} M) (Sigma, St. Louis, Missouri) (26). Colcemid (13.5×10^{-6} M) was added for 15 min before harvesting. Cells were collected, centrifuged, resuspended in 0.075 M KCl for 15 min at 37°C, and fixed three times in absolute methanol–glacial acetic acid (3:1). Air-dried chromosome preparations were used for in situ hybridization.

In Situ Hybridization. Molecularly cloned human oncogene probes for c-Ki-*ras* (exon 4B) and N-*ras* (exon 1) subcloned in PAT 153 and pBR322, respectively, were labeled with [^{3}H]TTP to high specific activity (2.2×10^7 cpm/μg) using an Amersham nick translocation kit. [^{3}H]DNA was separated from unincorporated nucleotides with an Elutip-d (Schleicher and Schuell, Keene, New Hampshire). The method described by Harper and Saunders (27) was utilized for hybridization of ^{3}H-labeled probes to the chromosomes. Prior to hybridization, slides were RNase-treated and denatured. Then 20 μl RNase (200 μg/ml in 2 × SSC) was applied to each slide, covered with a 24 × 50-mm coverslip, and incubated at 37°C for 1 h. The coverslips were removed and the slides washed four times with 2 × SSC at room temperature and dehydrated in an ethanol series. Chromosomal DNA was denatured by incubating the slides for 2 min at 70° in 70% formamide–2 × SSC. Slides were immersed in 30% ethanol at 0°C, followed by a dehydration series at room temperature.

The hybridization solution contained 1 μg/ml ^{3}H-labeled probe, 50% formamide, 10% dextran sulfate, 5 mM EDTA, 0.04% polyvinylpyrrolidone, 0.04% Ficoll, 0.04% bovine serum albumin, 300 mM sodium chloride, 30 mM sodium citrate, 50 μg/ml sonicated single-stranded salmon sperm DNA, and 20 mM phosphate buffer, pH 6.4. Hybridization solution was incubated at 70°C for 5 min to denature the probe and then quickly chilled. Then 25 μl was applied to each slide; the slides were covered with coverslips and incubated in a humid environment at 42°C for 24 h. Slides were washed once at room temperature for 20 min in 50% formamide–2 × SSC solution, four times for 5 min each in 2 × SSC, and dehydrated in an ethanol series.

Grain Localization. Slides processed for in situ hybridization were covered with NTB2 nuclear track emulsion (Kodak, Rochester, New York) diluted 1:1 with distilled water, and stored desiccated at 4°C for 14 days. Autoradiographs were developed for 4 min at 15°C in D19 developer, rinsed briefly in water, and fixed for 5 min. Air-dried slides were stained with 0.25% Wright stain in 0.06 M phosphate buffer, pH 6.8 (1:3) (28), and spreads showing grains located on chromosomes were photographed using an 80× objective (Zeiss Epiplan) without oil. To improve the quality of G-banding, slides were destained through an alcohol series, exposed to a solution of 0.03% trypsin–0.12% EDTA (Gibco Laboratories) in HBSS at pH 6.8 for 3 min, rinsed with water, and restained for 4–5 min with Wright stain. The previously photographed spreads were then relocated, photographed, and the grains localized on duplicate

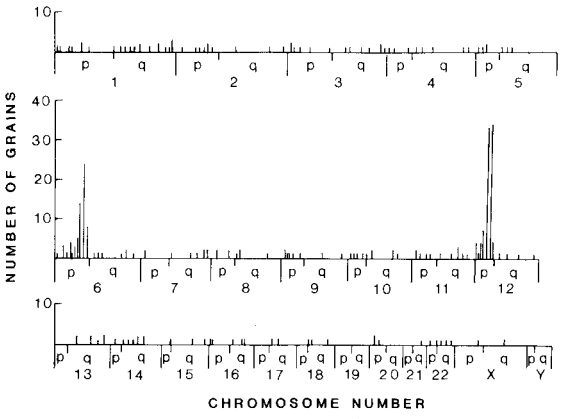

Fig. 1. Histogram representing silver grain distribution in 107 cells hybridized to a c-Ki-*ras*-2 probe which also has homology with the c-Ki-*ras*-1 locus. The number of grains is plotted against location on a 400-band human ideogram.

photomicrographs of chromosome spreads prior to and after G-banding.

RESULTS

The c-Ki-*ras* probe contains exon 4B of the c-Ki-*ras*-2 gene (11, 13, 25). The v-Ki-*ras*-related region in this 2.4-kb EcoRI fragment is also homologous to the c-Ki-*ras*-1 locus (13, 25). This c-Ki-*ras* probe detected homologous sequences, near the centromeres, in the short arms of chromosomes 6 and 12. The grain distribution of 107 cells examined in two experiments is represented as a histogram of a 400-band ideogram (29) (Fig. 1). Of a total of 308 grains, 71 (23%) and 91 (30%) clustered on chromosomes 6 and 12, respectively. The largest accumulation of grains was noted at bands 6p11-12 (46 grains) and 12p11.2-12.1 (70 grains) representing 38% of the total grains observed. Our previous studies (25) demonstrated that the c-Ki-*ras*-2 probe could readily distinguish restriction enzyme fragments characteristic of c-Ki-*ras*-1 and c-Ki-*ras*-2 (9, 13, 14). Thus, by employing human–rodent somatic cell hybrids containing either human chromosomes 6 or 12, it was possible to assign c-Ki-*ras*-1 and c-Ki-*ras*-2 to chromosomes 6 and 12, respectively. Therefore, the c-Ki-*ras*-1 locus is assigned by our in situ hybridization results to chromo-

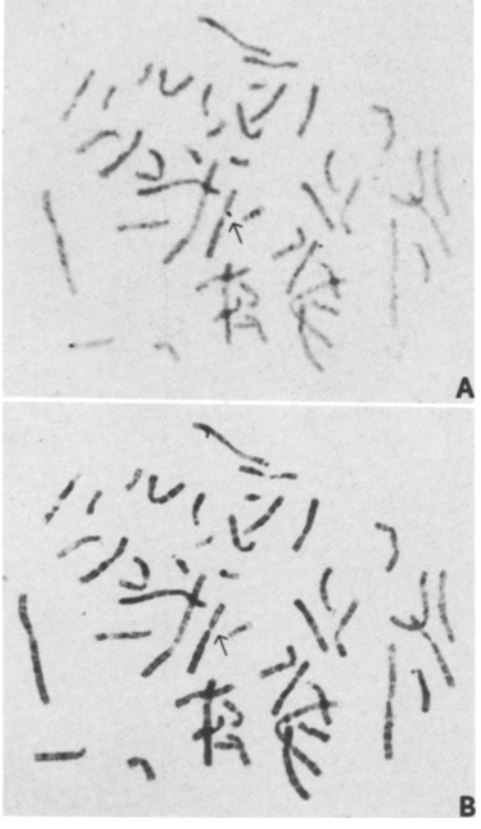

Fig. 2. Sequential staining of human mitotic chromosomes after in situ hybridization to a c-Ki-*ras* probe: (A) Wright-stained chromosomes with silver grains, and (B) G-bands after treatment with trypsin–EDTA and restained. Arrows indicate grain localized to 6p11-12.

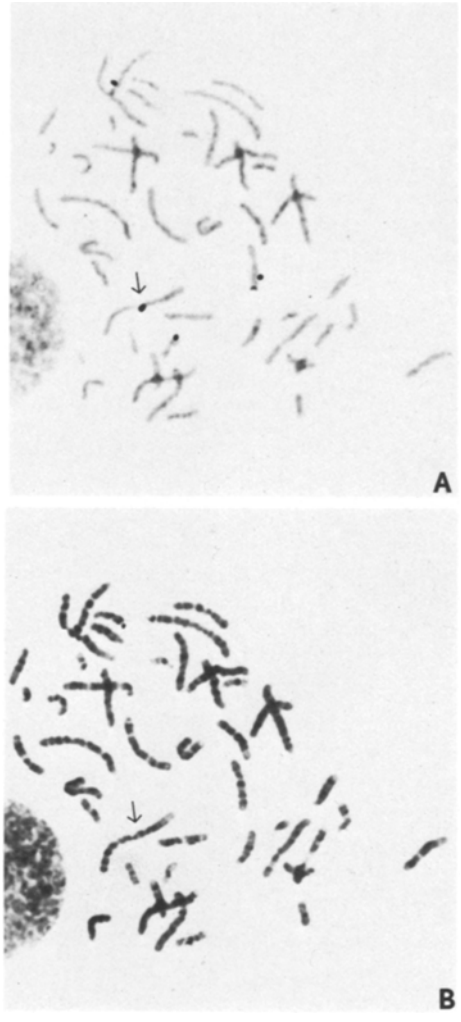

Fig. 3. Sequential staining of human mitotic chromosomes after in situ hybridization to a c-Ki-*ras* probe: (A) Wright-stained chromosomes with silver grains, and (B) G-bands after treatment with trypsin–EDTA and restained. Arrows indicate grain localized to 12p11.1.

some bands 6p11-12 and the c-Ki-*ras*-2 locus to 12p11.2-12.1. Chromosome spreads exhibiting label at the c-Ki-*ras*-1 and c-Ki-*ras*-2 loci on chromosomes 6 and 12 are illustrated in Figs. 2 and 3, respectively. Ten percent of the chromosome spreads examined exhibited label at both loci on chromosomes 6 and 12 (Fig. 4). Grains at sites other than those on chromosomes 6 and 12 were distributed randomly over the rest of the chromosomes.

The N-*ras* probe, a 0.9-kb Pvu II fragment, contains the first exon of this gene (8). In situ hybridization with this probe showed a significant accumulation of grains on the short arm of chromosome 1 in the vicinity of the centromere (Fig. 5). Of a total of 299 grains from 100 cells examined, 121 (40%) were on chromosome 1. The region 1p11-13 was the site with the largest accumulation of grains, exhibiting 78 (26%) of the total grains and 64% of the grains on chromosome 1. No other grain clustering was observed on other chromosomes. A metaphase photographed prior to and after G-banding with grains located at 1p11p-13 is shown in Fig. 6. Thus, N-*ras* is assigned to 1p11-13.

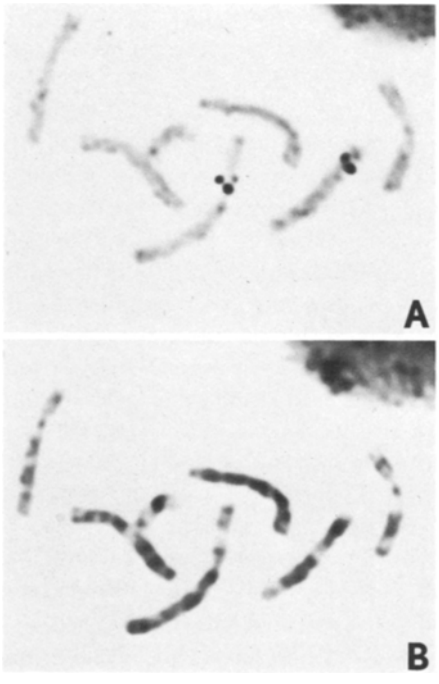

Fig. 4. Partial chromosome spread showing label on both chromosomes 6 and 12: (A) silver grains observed after in situ hybridization using a c-Ki-*ras*-2 probe which also has homology to a c-Ki-*ras*-1; (B) G-banding produced after subsequent treatment, permitting localization of grains to bands 6p11-12 and 12p11.1-12.1.

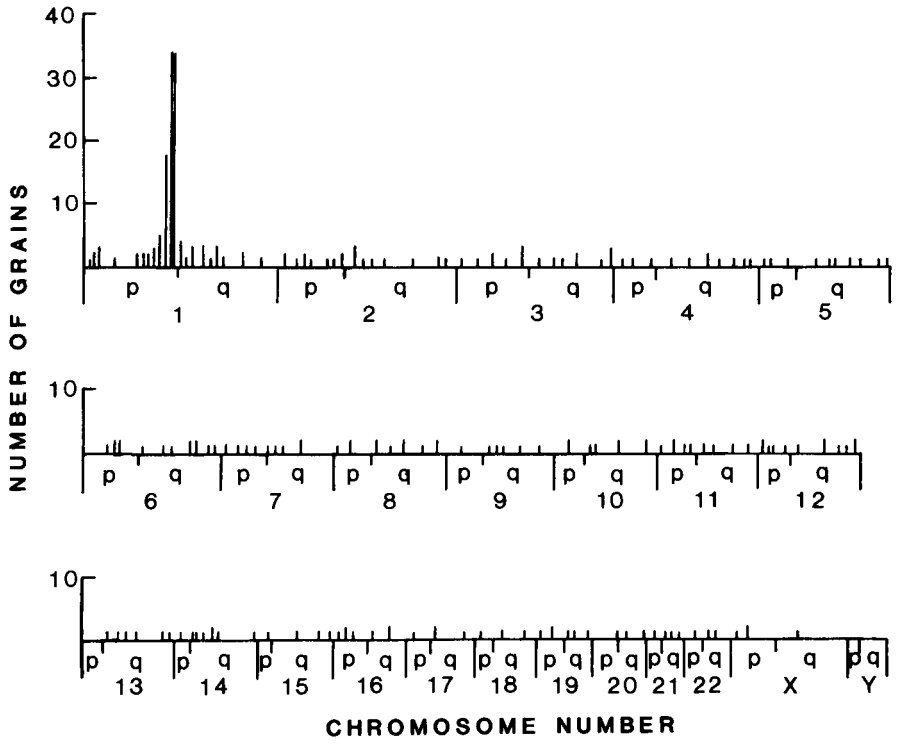

Fig. 5. Histogram representing silver grain distribution in 100 cells hybridized to a N-*ras* probe. The number of grains is plotted against location on a 400-band human ideogram.

DISCUSSION

The precise localization of c-Ki-*ras*-1 and c-Ki-*ras*-2 has not been possible using somatic cell hybrids and blot hybridization (25, 30–32) or by in situ hybridization using a v-Ki-*ras* probe (33). In the latter study with human pachytene chromosomes, a v-Ki-*ras* probe hybridized at two sites on chromosome 12, 12p12.1 and 12q24.2, but no *ras* sequences were identified on chromosome 6 (33). The present study, which employed in situ hybridization, demonstrates that c-Ki-*ras*-1 and c-Ki-*ras*-2 reside on chromosome bands 6p11-12 and 12p11.1-12.1, respectively. Interestingly, c-Ha-*ras*-1 is located on the short arm of chromosome 11 (34–36). Chromosomes 11 and 12 have distinctive banding pattern similarities and are probably ancestral homologs (37). In studies with cell hybrids and blot hybridization, N-*ras* was assigned to chromosome 1 (25, 38, 39). The current analysis confirms the location of N-*ras* at 1p11-13 (40, 41).

Although *ras* genes are activated by specific point mutations (15–20), the question arises as to whether chromosomal changes can also be responsible for activation of this family of human protooncogenes. Abnormalities of chromosomes 1, 6, and 12 have been identified in several forms of cancer (42–45). Common chromosome changes in neuroblastoma are deletions on the chromosome 1 short arm, homogeneously staining regions (HSR) or double minutes (DM) (46). Various deletions within 1p31-36 in neuroblastoma (47–50) are distant from the N-*ras*-locus and an association between this alteration, structure, and expression of the N-*ras* gene is not obvious. However, this does not exclude the possibility

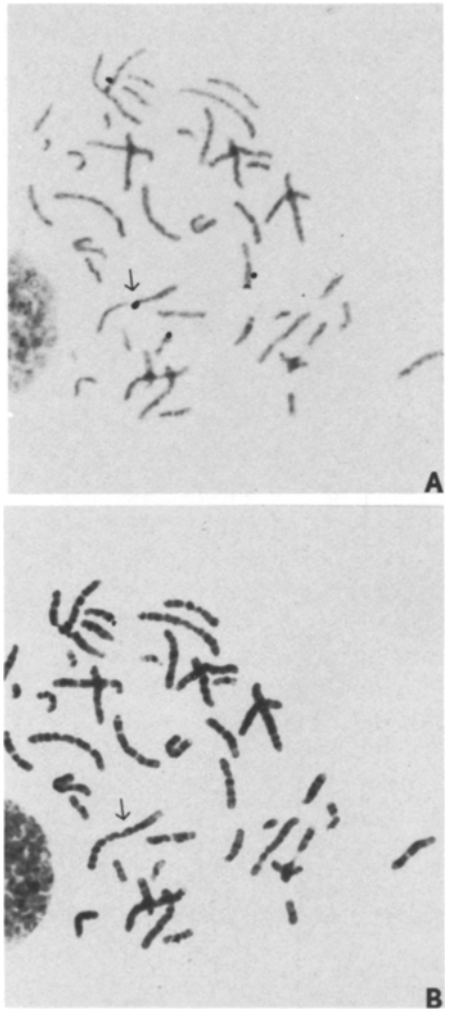

Fig. 6. Sequential staining of human mitotic chromosomes after in situ hybridization to a N-*ras* probe: (A) Wright-stained chromosomes with silver grains, and (B) G-bands after treatment with trypsin–EDTA and restained. Arrows indicate grain localized to 1p11-13.

that N-*ras* sequences could be altered or amplified within HSR or DM (38).

The isochromosome 6p, i(6p), is a characteristic abnormality in retinoblastoma (51, 52). Interestingly, in several cases of malignant melanoma (MM), i(6p) or rearrangements involving 6p have also been identified (53, 54). Alterations of 6p in MM are considered important since the genes of the major histocompatibility complex including HLA are located on 6p (53, 54). MM is one of few human cancers that can be recognized by the immune system, resulting in increased antibody production (55, 56). HLA and c-Ki-*ras*-1 loci are in relatively close proximity (57) and a positive effect caused by alterations in the centromere of chromosome 6 or its short arm could alter the expression of both genes. In animal systems, c-Ki-*ras* protooncogene expression is influenced by structural chromosome alterations. In a mouse adenocortical tumor, a c-Ki-*ras* protooncogene has been found amplified 30 to 60-fold and was located in an HSR and DM chromosomes (58). Changes involving chromosome 12, where the c-Ki-*ras*-2 proto-oncogene is located, were also reported; trisomy of chromosome 12 occurs in chronic lymphocytic leukemia (44, 45), and recently a translocation between chromosomes 11 and 12 has been found in a case of Wilm's tumor (59). Therefore, several human cancers exhibit alterations of chromosomes where c-Ki-*ras*-1 and c-Ki-*ras*-2 genes reside. Such human cancers should be examined to define the relationship between chromosome changes and Ki-*ras* protooncogene structures and activities.

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