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Genetics

Actively transcribed genes in the *raf* oncogene group, located on the X chromosome in mouse and human

(gene mapping/A-*raf* loci/*in situ* hybridization)

KAY HUEBNER*, ABBAS AR-RUSHDI*, CONSTANCE A. GRIFFIN†, MASAHARU ISOBE*, CHRISTINE KOZAK‡, BEVERLY S. EMANUEL†, LALITHA NAGARAJAN*, JOHN L. CLEVELAND§, TOM I. BONNER¶, MINDY D. GOLDSBOROUGH§, CARLO M. CROCE*, AND ULF RAPP§

*Wistar Institute of Anatomy and Biology, Philadelphia, PA 19104; †Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; ‡Laboratory of Viral Diseases, National Institute of Allergy and Infection Diseases, and ¶Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD 20892; and §Laboratory of Viral Carcinogenesis, Division of Cancer Etiology, National Cancer Institute, National Institutes of Health, Frederick Cancer Research Facility, Frederick, MD 21701

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ABSTRACT **Murine and human cDNAs, related to but distinct from c-*raf-1*, have been isolated and designated mA-raf and hA-raf, respectively. The mA-raf and hA-raf cDNAs detect the same murine and human fragments in Southern blots of restriction enzyme-cleaved murine and human cellular DNA. The murine restriction enzyme fragments homologous to mA-raf cDNA cosegregate with mouse chromosome X in a panel of Chinese hamster–mouse hybrid cells, thus localizing the mA-*raf* locus to mouse chromosome X. Two independently segregating loci, detected by the hA-raf cDNA (or mA-raf cDNA), hA-*raf-1* and hA-*raf-2*, are located on human chromosomes X and 7, respectively. The mA-*raf* locus and the hA-*raf-1* locus are actively transcribed in several mouse and human cell lines.**

The normal precursors to activated oncogenes, the protooncogenes, presumably serve important growth-regulatory functions, since at least 4 of the more than 25 known oncogenes derive from components of the signal transduction pathway of growth factors: *erbB*, which derives from a portion of the receptor gene for epidermal growth factor (1); *sis*, which is homologous to part of the gene for platelet-derived growth factor (2–4); the cellular homolog of the McDonough feline sarcoma oncogene, c-*fms*, which is related to the receptor for the colony-stimulating factor CSF-1 (5); and *myc*, which can mediate signal transduction for a variety of growth factors (6–8). Most oncogenes have been isolated as part of the genomes of transducing retroviruses (9–12), by molecular cloning of retrovirus integration sites in virus-induced tumor cells (13), or by DNA transfection using tumor-cell DNA (14). Additional oncogene-related genes (N-*myc* and r-*fos*) were identified by virtue of their nucleic acid sequence relatedness to known oncogenes (15–17), and putative oncogenes have been identified by virtue of their presence at the junction between chromosomes involved in specific translocations in leukemia and lymphoma (18–20).

The v-*raf* oncogene was originally isolated as part of the genome of 3611 murine sarcoma virus, an acutely transforming murine retrovirus (21). Subsequently, the avian homolog of v-*raf*, v-*mil* (or *mht*) was identified in the avian carcinoma virus MH2 (22, 23). v-*raf* and v-*mil* derive from the 3′-terminal two-thirds of the same cellular gene, c-*raf-1*. c-*raf-1* encodes a 74-kDa protein that is predominantly cytoplasmic (24). The deduced amino acid sequence of c-*raf-1* shows a distant relationship to the *src* family of oncogenes (25); however, in contrast to many *src*-family gene products, which have tyrosine-specific protein kinase activity, the proteins encoded by transforming versions of c-*raf-1* have protein kinase activity with specificity for serine or threonine residues (26). A role of c-*raf-1* in human tumors is suggested by its chromosomal location at 3p25 (27), a region that is commonly altered in small-cell lung carcinoma (28). Moreover, activation of c-*raf-1* occurs in primary stomach cancer (29).

Recently, on screening a mouse cDNA library with a v-*raf* oncogene probe, we have isolated a transforming *raf*-related cDNA, A-raf, that represents a gene distinct from c-*raf-1*. As an initial step in the analysis of this c-*raf-1*-related cDNA, a human A-raf cDNA has also been isolated, and the human and mouse A-raf cDNAs have been used to determine the chromosomal locations of A-*raf* genes in the mouse and human genomes and to assess activity of A-*raf* genes in various mouse and human cell lines.

MATERIALS AND METHODS

***raf*-Related cDNAs.** A cDNA library from B10.A mouse spleen cells (30) was screened with the *Xho* I–*Sph* I probe from v-*raf* (21), using moderate conditions of hybridization stringency [2× standard saline citrate (SSC), 60°C; 1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7.0]. A 1.6-kilobase (kb) cDNA clone was isolated that differed strikingly from v-*raf* in its restriction map and pattern of hybridization to various portions of the v-*raf* and human c-*raf-1* gene. Nucleic acid sequencing revealed homology of 69% between v-*raf* and the corresponding region of the cDNA and a 74% homology of their deduced amino acid sequences. The murine cDNA sequence, subcloned into the *Eco*RI site of pUC19, is subsequently referred to as mA-raf, and is more closely related to human c-*raf-1* than to v-*raf*, with 70% nucleic acid and 76% deduced amino acid sequence homology (31). A human A-*raf*-related cDNA, hA-raf, was isolated by screening a human fetal liver cDNA library with the mA-raf probe (hybridization conditions: 1× SSC, 60°C). The 1.9-kb hA-raf cDNA, subcloned into the *Eco*RI site of pUC12, has, on average, 90% nucleic acid sequence homology to the mA-raf cDNA in the shared coding region (U.R., unpublished results).

Southern Blot Analysis. DNAs from human, mouse, Chinese hamster, mouse–human hybrids, and Chinese hamster–mouse hybrids were prepared by cell lysis, proteinase K digestion, phenol extraction, and ethanol precipitation. Cellular DNA samples were digested with *Bgl* II (for Chinese hamster–mouse hybrids) or *Bcl* I (for mouse–human hybrids), fractionated in 0.8% agarose gels, and transferred to nitrocellulose or nylon filters as described by Southern (32).

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Abbreviation: kb, kilobase(s).

Hybridization was carried out at 42°C for 16 hr in 30% or 50% (vol/vol) formamide/4× SSC/0.02% Ficoll/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone containing sonicated salmon sperm DNA at 0.2 mg/ml. The probes were labeled by nick-translation. After hybridization, the filters were washed and exposed to Kodak XAR-5 film with an intensifying screen.

Cell Lines. Isolation, propagation, and characterization of parental cells and the mouse–human somatic-cell hybrids used in this study have been described (33–37). The Chinese hamster–mouse panel used for mapping the murine A-*raf* gene has also been described (38–40).

For regional localization of a human A-*raf* gene, two additional hybrid DNAs were used: DNA from a mouse–human hybrid retaining only the human X chromosome (41), designated hybrid 91B HAT, was kindly provided by B. Migeon; and DNA from a rat–human hybrid (42, 43), 52–63c117, retaining a translocation chromosome, t(14;X) (14pter→14q32.1::Xq13.1→Xqter), that includes most of human chromosome 14 plus most of the long arm of the human X chromosome.

Chromosomal *in Situ* Hybridization. Total 1.9-kb hA-raf cDNA-containing plasmid was nick-translated with all four ^{3}H-labeled dNTPs and hybridized to human metaphase chromosome preparations from peripheral blood cells of a normal man. The techniques used for *in situ* hybridization were essentially as described by Harper and Saunders (44). Human metaphases were hybridized at 37°C for 15 hr in 50% formamide/2× SSC/10% dextran sulfate containing sonicated salmon sperm DNA at 100 μg/ml. Autoradiography was performed using NTB-2 Kodak emulsion for 10–16 days before development. The slides were stained (45) for 5 min in a mixture of six parts of borate buffer (pH 9.2) to one part of Wright–Giemsa stain solution.

RNA Blot Analysis. For analysis of *raf*-related transcripts in human and mouse cell lines and hybrid cells, total cytoplasmic RNA was extracted as described (46). RNA was denatured in 50% formamide/2.2 M formaldehyde, electrophoresed in 1% agarose, and transferred to nitrocellulose membrane. Prehybridization and hybridization to nick-translated hA-raf cDNA were performed as described by Thomas (47).

Isolation and characterization of the mouse–human hybrids of T-cell phenotype have been described (35, 37). The human leukemia parental cells for one set of hybrids were peripheral blood lymphocytes from a patient with acute lymphocytic leukemia (patient no. 4, ref. 35).

RESULTS

The Murine A-*raf* Locus Is on Mouse Chromosome X. In *Bgl* II digests of mouse cellular DNA, the mA-raf cDNA detects bands at 4.7 and 1.4 kb, whereas Chinese hamster bands are 7.6 and 3.0 kb. The mA-raf cDNA clone was used to test, by Southern blot (32) analysis, for the presence or absence of the murine A-*raf* gene in *Bgl* II digests of cellular DNA from a panel of well-characterized Chinese hamster–mouse hybrids (38–40) retaining different complements of mouse chromosomes. Results of these hybridizations are summarized in Table 1 and demonstrate cosegregation of the mouse X chromosome with the mouse A-*raf* gene.

A-raf cDNAs Detect Loci on Human Chromosomes 7 and X. DNAs from a panel of well-characterized mouse–human hybrids retaining different subsets of human chromosomes were cleaved with *Bcl* I, fractionated, blotted, and hybridized initially to the ^{32}P-labeled 1.6-kb mA-raf cDNA probe under nonstringent conditions (30% formamide). The mA-raf cDNA detected two bands in *Bcl* I-digested mouse DNA (Fig. 1, lane 1), at 9.0 and 6.3 kb. In *Bcl* I-digested human DNA (lane 2), two bands (19 and 5.5 kb) are observed that hybridize with the mA-raf probe. The lower human band (5.5 kb), hereafter referred to as hA-*raf-2*, correlates with the presence of human chromosome 7 in the mouse-human hybrids, as can be seen immediately from Fig. 1; lane 4 contains DNA from a hybrid that retains only 7p11.4→7qter (33) (and all mouse chromosomes) and is positive for the 5.5-kb hA-*raf-2* fragment. The upper band (19 kb) seen in *Bcl* I digests of human DNA (lane 2) and some hybrid DNAs (lane 6) segregates independently of the 5.5-kb hA-*raf-2*, indicating the presence of a second A-*raf*-related locus, designated hA-*raf-1*, in the human genome. Hybridization of hA-raf cDNA (or mA-raf cDNA) to Southern blots of *Bcl* I-digested

Table 1. Correlation of presence of A-*raf* gene and specific mouse chromosomes in Chinese hamster–mouse hybrids

Mouse chromosome	No. of hybrid clones with A-*raf*/chromosome retention +/+	−/−	+/−	−/+	% discordant
1	9	4	4	5	41
2	8	7	5	2	32
3	5	6	1	2	21
4	5	7	8	2	45
5	2	7	12	2	61
6	10	7	4	2	26
7	10	5	3	4	32
8	6	8	7	1	36
9	5	9	7	0	33
10	3	9	10	0	45
11	0	8	9	0	53
12	5	5	1	3	29
13	2	7	4	1	36
14	5	9	8	0	36
15	6	3	0	5	36
16	4	6	2	2	29
17	9	5	0	3	18
18	4	6	2	2	29
19	9	7	4	1	24
X	13	9	0	0	0

DNA (10 μg) from Chinese hamster–mouse hybrids was digested with an excess of restriction endonuclease, fractionated by electrophoresis, and blotted. Filters were hybridized with nick-translated ^{32}P-labeled mA-raf cDNA probe. The total number of hybrids tested for concordance between A-*raf* gene presence and chromosome retention differs for specific chromosomes because not all hybrids were characterized for the presence of every mouse chromosome.

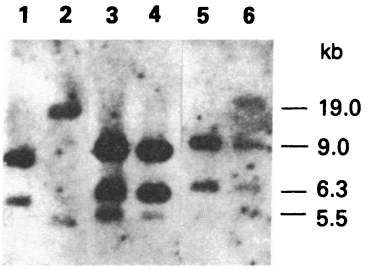

FIG. 1. Detection of human *raf*-related loci in mouse–human hybrids by use of mA-raf cDNA. Cellular DNA (10 μg per lane) was cleaved with restriction enzyme *Bcl* I, fractionated by electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled murine A-raf cDNA. Lane 1: mouse cell line (NP3 BALB/c myeloma). Lane 2: human peripheral blood lymphocytes. Lane 3: mouse–human hybrid N9, containing human chromosomes 6, 7, 17q, and 21. Lane 4: mouse–human hybrid C136is, containing human chromosome 7p11.4→7qter. Lane 5: mouse–human hybrid 8C, containing human chromosomes 5, 8, 9, 12, 13, 14, 15, 17, 21, and 22. Lane 6: mouse–human hybrid CSK-12, containing human chromosomes 1, 2, 3, 4, 6, 7, 9, 11, 12, 14, 15, 18, 20, 21, 22, and X. Approximate lengths of mouse and human DNA fragments that hybridized with the probe are given at right.

hybrid DNAs indicated a correlation between presence of the human X chromosome and the 19-kb hA-*raf-1 Bcl* I fragment (Fig. 2).

A summary of all data obtained by Southern blot analysis of the mouse–human hybrid panel probed with murine and human A-raf cDNAs is shown in Fig. 3. The presence of human chromosome 7 correlated with the presence of the hA-*raf-2* locus, and the presence of the human X chromosome correlated with the presence of the hA-*raf-1* locus. In addition to the hybrids listed in Fig. 3, DNA from a hybrid, 91B HAT, retaining only human chromosome X (41) retained the hA-*raf-1* gene but not the hA-*raf-2* gene; and a hybrid, 52-63c117, retaining most of the long arm of the human X chromosome was negative for hA-*raf-1*, indicating that the hA-*raf-1* gene is localized to the chromosome region Xpter→Xq13.1.

hA-*raf-2* Is at 7p14→7q21 and hA-*raf-1* at Xp21→Xq11. To determine more precisely the locations of the A-*raf* genes on human chromosomes, *in situ* hybridization was performed using the hA-raf cDNA. Nick-translated ^{3}H-labeled plasmid containing the 1.9-kb hA-raf cDNA was hybridized to metaphase chromosomal preparations from a normal man. The combined data for three experiments showed that in a total of 178 metaphases containing 340 chromosomally localized grains, 67 grains (20%) were localized to chromosome 7, and 38 grains (11%) were localized to the X chromosome. As can be seen in Fig. 4, 51 grains were located in region 7p14→7q21 and 33 grains were located in region Xp21→Xq11, with most grains at Xp11→Xp13. No other region of similar length showed significant hybridization with the hA-raf probe. Region 7p14–7q21 represents about 2% of the haploid autosome length. Our finding that 15% of all grains are located on this region is significant, and this site had almost 4 times as many grains as did the next highest site aside from the location on X. Since one hybrid containing only human chromosome region 7p11.4→7qter retained ha-*raf-2* (Fig. 1, lane 4), the localization of hA-*raf-2* can be narrowed to 7p11.4→7q21. Region Xp21→Xq11 also represents about 2% of the haploid autosomal length, and the finding that 10% of all grains are located on this region is significant; this region had about 6 times as many grains as did the next most frequent site, when one corrects for the fact that there is only one X chromosome in each metaphase spread scored. These data, in conjunction with the somatic-cell hybrid results, suggest that hA-*raf-1* and the related hA-*raf-2* gene map to the pericentromeric regions of chromosomes X and 7, respectively.

Expression of *raf*-Related Genes in Murine and Human Cells. The murine and human A-raf cDNAs were selected from murine spleen and human fetal liver cDNA libraries, respectively; thus A-raf mRNAs are present in at least these two tissues. The murine A-*raf* locus has also been found to be expressed as an mRNA of ≈2.6 kb in mouse fibroblasts and cells of myeloid and T-cell lineage (data not shown). Since there were two c-*raf-1*-related loci, hA-*raf-1* and hA-*raf-2*, in the human genome, it was of interest to determine whether one or both of these loci represent active genes. Blot hybridization of electrophoretically fractionated cytoplasmic RNA from various cell lines was performed using the

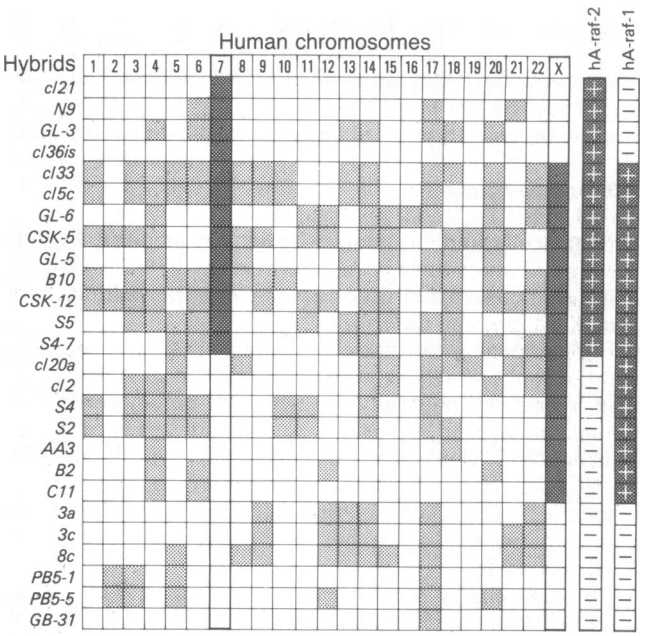

FIG. 3. Presence of human A-*raf* genes in a panel of 26 mouse–human hybrids. Stippling indicates that the hybrid clone named at left contains the chromosome indicated at top. Columns for human chromosomes 7 and X are boldly outlined to emphasize the correlation of the presence of human chromosomes 7 and X with the presence of hA-*raf-2* and hA-*raf-1*, respectively.

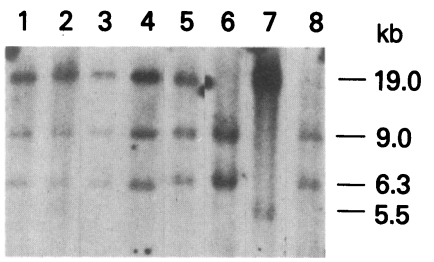

FIG. 2. Detection of human *raf*-related loci in mouse–human hybrids by use of hA-raf cDNA. *Bcl* I-digested DNA (10 μg per lane) was from mouse–human hybrids retaining human chromosomes 5, 6, 13, 14, 18, 20, 22, and X (lane 1); 3, 4, 5, 6, 7, 11, 13, 14, 15, 17, 18, and X (lane 2); 4, 6, and X (lane 3); 4, 6, 12, 20, and X (lane 4); and 4, 18, and X (lane 5); from a T-cell hybrid missing human chromosomes 7 and X (lane 6); from human peripheral blood lymphocytes (lane 7); and from a mouse cell line (NP3 BALB/c myeloma, lane 8). Approximate sizes of human and mouse DNA fragments detected with the hA-raf probe are the same as the bands detected with mA-raf cDNA (see Fig. 1).

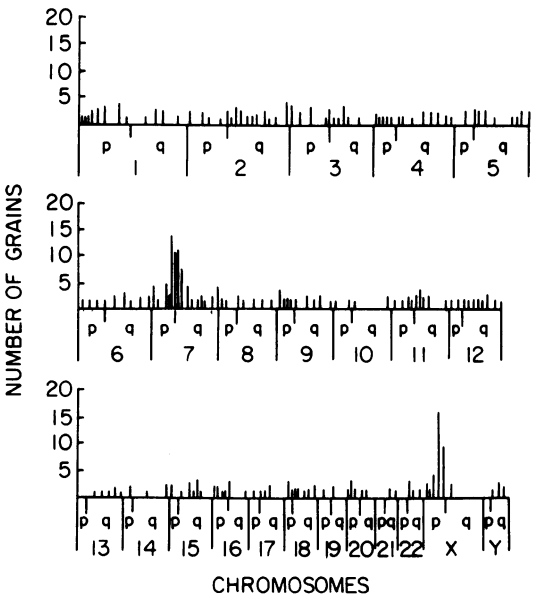

FIG. 4. *In situ* localization of hA-*raf-1* and hA-*raf-2* in the human genome using the hA-raf cDNA. Diagram shows the grain distribution on human chromosomes in 178 male metaphases. The abscissa represents the relative sizes of the respective human chromosomes; the ordinate represents the number of silver grains.

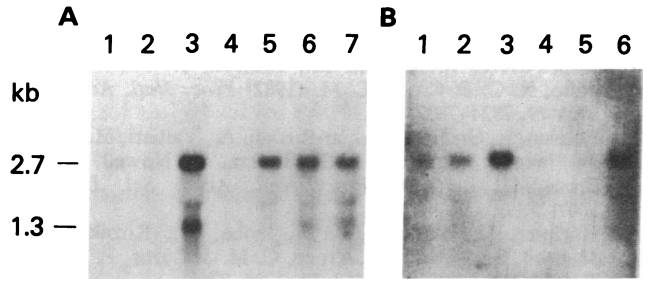

FIG. 5. Expression of hA-*raf* genes in human and hybrid cell lines. Total cytoplasmic RNA (20 μg per lane) was denatured, fractionated, transferred to nitrocellulose, and hybridized to the hA-raf cDNA probe. (*A*) Lane 1: mouse T-cell line BW5147. Lane 2: BW5147–Supt1 hybrid cell line that has lost human chromosomes 7 and X. Lane 3: human T-cell subclone of Supt1. Lane 4: BW5147–Supt1 hybrid cell line that has lost human chromosomes 7 and X (see Fig. 2, lane 6). Lane 5: BW5147–Supt1 hybrid cell line that retains human chromosome X but has lost human chromosome 7. Lane 6: human T-cell line Supt1 (48). Lane 7: human T-cell line Jurkat. (*B*) Lane 1: mouse T-cell line BW5147. Lane 2: BW5147–Supt1 T-cell hybrid retaining human chromosome X but not 7 (same hybrid as in *A*, lane 5). Lane 3: BW5147–human leukemia hybrid retaining human chromosomes 4, 18, and X (DNA for this hybrid is in Fig. 2, lane 5). Lane 4: hybrid between mouse thymidine kinase-deficient L cell and human fibroblast, retaining human chromosomes 4, 6, 7, 13, 14, 17, 18, and 20. Lane 5: hybrid between mouse macrophage and simian virus 40-transformed human fibroblast, retaining only human chromosome 7. Lane 6: human T-cell line Jurkat. Note that the 2.7-kb RNA correlates with the presence of the human X chromosome but not with the presence of human chromosome 7. The human 1.3-kb RNA species is present only in the human T cells and not in the hybrids and could conceivably represent an hA-*raf-2* transcript.

human A-raf cDNA as probe. In the sample from a murine T-cell line (Fig. 5*A*, lane 1), a faint band (slightly below 2.7 kb and which was also detected strongly by mA-raf cDNA when blots were washed and rehybridized) is seen after hybridization with the hA-raf cDNA and probably represents transcription of the murine A-*raf* locus. The human T-cell clones (Fig. 5*A*, lanes 3, 6, and 7) express a hA-*raf*-homologous RNA of 2.7 kb that is also seen in hybrid cells of T-cell phenotype that retain the human X chromosome (Fig. 5*A*, lane 5; Fig. 5*B*, lane 3) but not in T-cell hybrids (Fig. 5*A*, lanes 2 and 4) or fibroblastic hybrids (Fig. 5*B*, lanes 4 and 5) that have lost the human X chromosome. Fig. 5 also shows that fibroblastic hybrids retaining human chromosome 7 (but not X) do not express a human A-*raf*-related transcript. HL-60 promyelocytic leukemia cells also express a 2.7-kb A-*raf* transcript, as do hybrids between HL-60 and mouse myeloma cells that retain the human X chromosome (data not shown). In summary, the single X-linked murine A-*raf* locus is transcribed in murine fibroblasts and spleen, myeloid, and T cells (data not shown); the hA-*raf-1* locus on the human X chromosome is transcribed in human T cells, fetal liver cells, and HL-60 cells. We have not yet conclusively shown that the hA-*raf-2* locus on chromosome 7 is transcribed.

DISCUSSION

A murine cDNA clone, isolated from a mouse spleen cDNA library by homology to a v-*raf* probe, differed strikingly from v-*raf*. Nucleic acid sequence homology between the cDNA, referred to as mA-raf, and c-*raf-1* was 70%. The mA-raf cDNA was subsequently used to isolate a related human cDNA, hA-raf, and the murine and human cDNAs were employed to map the *raf*-related genomic loci in mouse and human and to analyze transcriptional activity of murine and human A-*raf* genes.

In Chinese hamster–mouse hybrids retaining various subsets of mouse chromosomes, the presence or absence of the single mA-*raf* locus was concordant with the presence or absence of the mouse X chromosome.

In screening mouse–human hybrids, retaining different groups of human chromosomes, with the mA-raf and hA-raf cDNAs, two human *raf* loci segregated concordantly with human chromosomes 7 and X, respectively. The hA-*raf-1* locus was shown by *in situ* hybridization to map to the region Xp21→Xq11 and the hA-*raf-2* locus to map to the region 7p11.4→7q21. The hA-*raf-2* locus on chromosome 7 is distinct from the epidermal growth factor receptor locus at 7p12→7p13 (49–51) and from the *MET-1* transforming gene at 7q21→q31 (33, 52). The hA-*raf-1* locus maps to an interesting region of the human X chromosome, Xp21→Xq11, near (in cytogenetic distances) the locus for the testicular feminization (TFM) syndrome (53) and Menkes syndrome (54), which are linked in mouse and human (55).

In the human, the order of markers mapped to the X chromosome in the relevant region is as follows: ornithine transcarbamylase (*OTC*) at Xp21; X-controlling element (*XCE*) at q11.2→q21.1; TFM syndrome and Menkes syndrome at p11→q13, phosphoglycerate kinase (*PGK*) at q13; and α-galactosidase (*GLA*) at q21→q24. An X-linked immunodeficiency locus (*XID*) may be located between *PGK* and *GLA* (q13→q22) in humans, if it is in a region similar to its location on the mouse X chromosome (56, 57). In the mouse, the order of homologous genes on the X chromosome is *xce, tfm, pgk*, mottled (mouse homolog of Menkes disease), *xid*, and *gla* (55). Although mA-*raf* was not regionally localized in the mouse, it is probable that it falls within this region. Thus, in mouse and human, the X-linked, actively transcribed A-*raf-1* gene is located in an interesting region of the X chromosome near *TFM* [which encodes a steroid receptor (58)], and perhaps near the immune deficiency locus, which could also represent a receptor gene or gene family.

In light of the recent spate of reports of linkage of the cystic fibrosis gene(s) to chromosome 7 (59–61), specifically to region centromere→7q21 (60, 61), the hA-*raf-2* gene is also in an interesting and possibly useful position on chromosome 7, though our study places hA-*raf-1* near the centromere, and *MET-1*, which is tightly linked to the cystic fibrosis locus (61), is presumably closer to 7q21 (52).

The single mouse A-*raf* locus is transcribed in mouse cell lines of myeloid and T-cell lineage. The hA-*raf-1* locus is actively transcribed in human cell lines of myeloid and T-cell lineage, as well as in mouse–human hybrids of B- and T-cell phenotype that retain the human X chromosome and not human chromosome 7. Transcripts of the hA-*raf-2* locus on chromosome 7 were not detected in fibroblast-like mouse–human hybrids retaining human chromosome 7. Determination of tissue specificity of expression of mouse and human A-*raf* loci will require further studies.

Although we have not yet functionally characterized the product of the A-*raf* loci, serine/threonine-specific protein kinase activity for the transforming proteins of c-*raf-1* has been detected (26). Because of the close homology of c-*raf-1* and A-raf cDNA in the kinase domain (80%) (62), we speculate that the A-*raf* gene product also has serine/threonine-specific kinase activity. Thus, the discovery of v-*raf*, a transforming version of c-*raf-1*, has allowed access to a family of potential oncogenes.

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