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12. Cultures were obtained from dissociated, arachnoid-free ventral spinal cord tissue prepared from rat embryos in the 14th to 15th day of development (stages 24 to 27) [G. A. Christie, *J. Morphol.* **114**, 263 (1964)]. The cells were plated onto polylysine-coated, 35-mm-diameter culture dishes at a concentration of 5×10^5 viable cells per dish in Dulbecco's modified Eagle's medium supplemented with 10 percent horse serum (inactivated by heating at 50°C for 30 minutes), 2 m*M* glutamine, 33 m*M* glucose, and 0.15 m*M* Garamycin. Cytosine arabinoside (10^{-5} *M*) was added to this medium for 12 to 24 hours 1 day after plating to retard the proliferation of nonneuronal cells and thereby reduce the supportive effect of glia [T. Meyer, W. Burkart, H. Jockusch, *Neurosci. Lett.* **11**, 59 (1979); R. L. Schnaar and A. E. Schaffner, *J. Neurosci.* **1**, 204 (1981)].
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Human c-Ki-*ras*2 Proto-Oncogene on Chromosome 12

Abstract. *A human colonic adenocarcinoma transforming gene, recently identified as a cellular homolog of the Kirsten sarcoma gene (v-*ras*), was used to assign the human cellular Kirsten* ras*2 gene to chromosome 12 by the Southern hybridization method. A single 640 base-pair Eco RI–Hind III fragment of the transforming gene, isolated by DNA transfection and molecular cloning, can detect a single Eco RI fragment (2.9 kilobase pairs) of DNA from phenotypically normal cells. The data suggest a constant chromosomal location of c-Ki-*ras*2.*

The use of DNA-mediated gene transfer techniques (*1*) to identify the origin of transforming sequences in malignant cells has provided evidence that changes in genes in the normal cell can give rise to activated forms with oncogenic potency [(*2–5*); see (*6*) for review]. This emerging class of cellular transforming sequences has been detected by a biological assay in which mouse NIH 3T3 cells incubated with DNA from tumor cell lines of different tissue origins acquire an altered morphology and the ability to grow in soft agar. These traits are often associated with malignant cells (*2–5*). NIH 3T3 cells treated with DNA from human tumor cell lines acquire human DNA that can be identified by means of a species-specific molecular probe (Alu) (*7*) that detects repetitive sequences interspersed in the human genome (*4, 5*). DNA from such mouse cell transformants can be used to construct a recombinant genomic library from which phage clones containing specific human transforming sequences can be isolated (*6, 8, 9*).

Combining human-rodent somatic cell hybrids and Southern (*10*) hybridization techniques [see (*11*) for review], we have chromosomally assigned the c-Ki-*ras*2 gene (*12*). The probe was a transforming gene isolated from the SW480 human colon carcinoma cell line by DNA-mediated transfection and molecular cloning (*5, 6*). A pBR322 plasmid clone (p640) containing a unique 640 bp Eco RI–Hind III fragment of the colon transforming gene was used as a probe for filter hybridization studies.

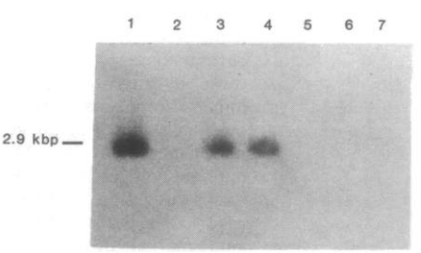

Fig. 1. The p640 colon transforming gene probe was hybridized to DNA's from human (lane 1), mouse (lane 2), and hybrid cells (lanes 3 to 7). The probe detects a single 2.9-kbp Eco RI fragment of human DNA. Under these hybridization conditions (*21*), mouse DNA does not anneal with the probe. Hybrid cell DNA's in lanes 3 and 4 are positive for c-Ki-*ras*2.

DNA's from human and mouse cell lines were cleaved with Eco RI, electrophoresed through agarose, and transferred onto nitrocellulose. The p640 plasmid was labeled with ^{32}P by nick translation (*13*) and hybridized to filters (Fig. 1). A single 2.9-kbp Eco RI human DNA fragment hybridized with the probe, whereas no signal was obtained from mouse DNA under these hybridization conditions. We then screened the DNA's from 38 man-mouse hybrid cell lines for the presence of c-Ki-*ras*2. At the same time, correlated homogenates of hybrids were tested for genetic markers for the 22 human autosomes and the X chromosome to determine their human chromosome complement. The detection of a 2.9-kbp fragment by the probe indicated the presence of the c-Ki-*ras*2 gene in the DNA of a hybrid cell line (Fig. 1, lanes 3 and 4). This 2.9-kbp DNA fragment was coordinately present in cell hybrids only with the human chromosome 12 markers, lactate dehydrogenase B and peptidase B (Fig. 2). The data have been summarized so that only the percent discordancy for each human chromosome is given in Fig. 2 (percent of hybrids in which c-Ki-*ras*2 and a given human chromosome marker did not cosegregate). In 13 karyotyped hybrids, c-Ki-*ras*2 segregated only with a normal human chromosome 12 (Fig. 2). The c-Ki-*ras*2 gene is therefore asyntenic with at least eight other human proto-oncogenes (*14*).

The hybrids used for assignment studies were derived from 12 unrelated individuals, indicating a constant chromosome location for c-Ki-*ras*2 in normal human cells. A survey of more than 20 DNA's from unrelated individuals revealed no Eco RI polymorphism in the region of the gene detected by the probe. Intensities of signals on x-ray film after hybridization of the p640 probe to these DNA's were similar, suggesting that the copy number of the gene is invariant (less than twofold), at least in the normal human fibroblasts and leukocytes examined. In addition, DNA's from HeLa, HEL (human embryonic lung), and RD (human rhabdomyosarcoma) cell lines yielded hybridization signals comparable to those obtained with normal cell DNA.

Several transforming genes detected by the NIH 3T3 transfection system bear

homology to certain oncogenes of acutely transforming retroviruses of rodents (*15, 16*). For example, the transforming gene isolated from human EJ bladder carcinoma cells is related to v-Ha-*ras*, the oncogene of Harvey sarcoma virus (*15, 16*). The colon transforming gene has been identified as c-Ki-*ras*2, and is thus related to the oncogene of Kirsten murine sarcoma virus (*15, 17*). Identical transforming genes have been identified in six different human colon and lung carcinoma cell lines (*4, 5, 15, 17*) as well as in a biopsy of a human adenocarcinoma of the colon (*17*). Therefore, in at least seven independently derived human tumor cells, cellular homologs of retroviral oncogenes have become activated.

The karyotypes of various tumors show distinct and well-characterized abnormalities. Examples of this are the alterations of chromosome 22 seen in chronic myelogenous leukemia, or the translocations involving chromosome 8 in Burkitt's lymphoma (*18*). Presumably, each of these changes reflects specific alterations at the molecular level. Thus, the activation of a proto-oncogene might be correlated with changes in associated chromatin or chromosomal regions. Consequently, the chromosome 12 locus now equated with c-Ki-*ras*2 may eventually be associated with observable karyotypic abnormalities in certain types of human tumors.

In the case of the SW480 colon carcinoma cell line, c-Ki-*ras*2 has undergone two kinds of alterations. The first involves a lesion which enables it to induce transformation of NIH 3T3 cells (*5*). This lesion, which created the colon transforming gene, may possibly involve a protein-encoding region of the gene (*19*). In addition, the oncogene has undergone amplification, such that it is present in these cells in three to five times normal amounts (*17*). These amplified genes may be related to the double-minute chromosomal particles that are present in comparable numbers in these cells. However, to date no observable alteration in karyotype of chromosome 12 has been found (*20*).

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	ICL-15	REW-8I CSAz4	WIL-14	XER-7	XER-11	TSL-2	ATR-13	JSR-17G	EXR-5 CSAz	DUM-13	NSL-9	NSL-15	NSL-16	% Discordancy (38 hybrids)
c-Ki-ras2	+	-	-	+	-	+	+	+	+	+	+	+	+	
Chromosomes*														
1	-	-	-	+	+	-	+	+	+	+	-	-	-	32
2	-	-	-	+	-	+	+	-	-	+	-	+	-	40
3	-	-	+	+	+	-	+	+	+	+	-	-	+	50
4	-	-	-	+	+	-	+	-	+	-	-	+	+	57
5	-	-	-	+	-	+	-	+	+	+	+	+	+	47
6	-	-	-	+	+	+	+	-	+	+	-	-	-	28
7	-	-	-	+	+	-	+	-	+	-	-	+	+	37
8	-	-	-	+	+	-	+	+	+	-	+	+	-	33
9	-	-	-	+	-	-	-	-	-	-	-	-	-	47
10	-	-	-	+	+	+	+	+	+	+	+	-	-	22
11	-	-	-	-	-	-	-	+	-	+	-	-	-	37
12	+	-	-	+	-	+	+	+	+	+	+	+	+	0
13	-	-	-	+	-	-	-	+	+	-	+	+	-	37
14	-	-	-	+	-	-	+	+	+	+	+	+	-	42
15	-	-	-	+	+	-	+	+	+	-	+	+	+	42
16	-	-	-	-	+	-	-	-	-	+	+	-	-	42
17	+	+	+	-	+	-	+	+	+	+	+	+	+	47
18	-	-	-	+	+	+	+	+	+	+	-	+	+	39
19	-	-	-	-	-	-	-	-	+	+	-	+	-	34
20	+	-	-	-	+	+	-	+	+	+	+	-	+	34
21	+	+	-	-	-	+	-	+	+	+	+	+	+	38
22	-	-	-	-	+	-	-	+	+	+	+	+	-	53
X	-	-	-	+	-	+	-	+	+	-	-	+	-	39

*The cell hybrid lines also contained the following chromosome translocations originating from the human parental cells used in cell fusions: XER-7, 11/X; XER-11, 11/X and X/11; TSL-2, 17/3; ATR-13, 5/X; JSR-17G, 7q$^-$; EXR-5 CSAz, X/11; DUM-13, X/15 and 15/X; NSL-9, 17/9; NSL-16, 17/9, and 9/17.

Fig. 2. Segregation of c-Ki-*ras*2 with human chromosomes and chromosome markers in man-mouse hybrids. The construction and characterization of these hybrid lines has been described (*11*). Chromosomes were scored after trypsin-Giemsa staining (*22*). A "+" indicates the presence of the indicated human chromosome in ≥ 10 percent of the metaphases scored. The segregation of c-Ki-*ras*2 in additional 38 man-mouse hybrid lines has been summarized in the right-hand column. The human chromosome content of these hybrids was determined by analysis of previously assigned human genetic markers (*11*). The percent discordancy for the segregation of c-Ki-*ras*2 and a given chromosome marker for the 38 cell hybrids is listed.

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12. The colon transforming gene has been shown to be homologous to v-Ki-*ras*, the oncogene of Kirsten murine sarcoma virus (*15, 17*). It is the normal human cellular homolog c-Ki-*ras*2, the progenitor of the colon transforming gene (*19*), that we now assign to chromosome 12. The nomenclature for cellular and retroviral oncogenes used in this report follows the system proposed by J. M. Coffin *et al.* [*J. Virol.* **40**, 953 (1981)].
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Spermidine Requirement for Cell Proliferation in Eukaryotic Cells: Structural Specificity and Quantitation

Abstract. *Six structural homologs of spermidine and five of its precursor, putrescine, were studied for their ability to prevent cytostasis of cultured L1210 leukemia cells induced by α-difluoromethylornithine (DFMO), a specific inhibitor of putrescine biosynthesis. High-performance liquid chromatography and competition studies with spermidine indicated that the homologs, which vary in the length of the carbon chain separating the amines, penetrated the cells. The structural specificity of the spermidine carrier was defined. Three of the six spermidine homologs supported cell growth during a 48-hour incubation in the presence of DFMO, indicating that a two-carbon extension of spermidine structure was tolerated for biological function. Two of the five putrescine homologs supported growth after being converted by the cells to their respective spermidine homologs. The central nitrogen of spermidine appears to be essential for function since diamines of chain length comparable to that of spermidine did not prevent DFMO cytostasis. No more than 15 percent of the spermidine normally present in L1210 cells was required for cell proliferation in the presence of DFMO.*

Several lines of evidence indicate that polyamines, particularly spermidine, are required for cell proliferation: (i) they are found in greater amounts in growing than in nongrowing tissues (*1*); (ii) prokaryotic and eukaryotic mutants deficient in polyamine biosynthesis are auxotrophic for polyamines (*2*); and (iii) inhibitors specific for polyamine biosynthesis also inhibit cell growth (*3*, *4*). Despite this evidence, the precise biological role of polyamines in cell proliferation is uncertain. It has been suggested that polyamines, by virtue of their charged nature under physiological conditions and their conformational flexibility, might serve to stabilize macromolecules such as nucleic acids by anion neutralization (*5*, *6*). Any specificity of such interactions would probably reside in the spatial separation of the amines to ensure optimal reactivity at their site of action. We now report the results of a study undertaken to quantitate and define structurally the spermidine requirement of eukaryotic cell proliferation.

Five putrescine and six spermidine homologs of various aliphatic chain lengths were examined for their ability to prevent cytostasis induced in cultured L1210 cells by α-difluoromethylornithine (DFMO). The latter is a highly specific and irreversible inhibitor of ornithine decarboxylase (*4*), which is the initial enzyme in putrescine and spermidine biosynthesis. Treatment of cells with DFMO results in a depletion of intracellular putrescine and spermidine pools, followed by cytostasis. The cytostasis can be readily prevented by including spermidine or its precursor, putrescine, in the DFMO-containing medium. If the medium is supplemented instead with various homologs of spermidine or putrescine, the structural constraints of the biological sites critical for cell proliferation can be mapped, provided that the homologs enter the cells. This test system may not be entirely equivalent to the situation in untreated cells since DFMO results in a marked increase in decarboxylated *S*-adenosylmethionine and *S*-adenosylmethionine decarboxylase (*7*), the enzyme required for the conversion of putrescine to spermidine.

A series of putrescine homologs having the general structure $NH_2(CH_2)_nNH_2$ [abbreviated, DA_n (for diamine), with DA_4 being putrescine itself (Table 1)] and spermidine homologs having the general structure $NH_2(CH_2)_nNH(CH_2)_{n'}NH_2$ [abbreviated, $_nTA_{n'}$ (for triamine), with $_3TA_4$ being spermidine itself] were accrued (*8*). Their ability to enter L1210 cells was evaluated according to three separate parameters.

1) The spermidine homologs competed effectively with [^{3}H]spermidine for uptake (Table 1). Although most of the spermidine homologs were in the same range of effectiveness, homospermidine ($_4TA_4$) was clearly the most competitive, with an inhibition constant (K_i) of 3.5. The slightly better competition of $_3TA_8$ over other long-chain spermidine homologs could be due to its resemblance, in chain length, to spermine. The putrescine homologs competed poorly with spermidine, and their effectiveness

Table 1. Inhibition of [^{3}H]spermidine uptake into L1210 cells by polyamines or their homologs. The abbreviation for putrescine homologs having the general structure $NH_2(CH_2)_nNH_2$ is DA_n (for diamine) where n is 3 to 8. The abbreviation for spermidine homologs having the general structure $NH_2(CH_2)_nNH(CH_2)_{n'}NH_2$ is $_nTA_{n'}$ (for triamine) where n is 3 or 4 and n' is 3 to 8.

Homolog	K_i* (μM)	[^{3}H]Spermidine uptake inhibition†	
		Picomoles per 10^7 cells-min	Percent of control
None		56.1	100
DA_3	> 500	54.0	96
DA_4 (putrescine)	171.3	44.6	80
DA_5	459.0	54.0	96
DA_6	63.2	40.1	71
DA_7	18.2	23.0	41
DA_8	22.1	25.2	45
$_3TA_3$	8.4	16.1	29
$_4TA_4$	3.5	7.3	13
$_3TA_5$	12.3	19.8	35
$_3TA_6$	13.1	19.6	35
$_3TA_7$	13.0	20.0	36
$_3TA_8$	7.8	13.5	24
Spermine	9.1	17.1	30

*Prewarmed L1210 cells (5×10^6) were incubated for 20 minutes in 1 ml of RPMI-1640 media containing 2 percent Hepes-Mops and 0.2, 0.5, 1.0, 2.0, 5.0, or 10 μM [^{3}H]spermidine and 100 μM homolog. Uptake data were fitted by computer for competitive inhibition; the Michaelis constant for spermidine uptake was 2.0 μM, and the maximum velocity of the reaction was 117 pmole/min per milligram of protein. †Cells were incubated for 20 minutes at 37°C with 10 μM [^{3}H]spermidine plus 100 μM putrescine or spermidine homolog.