Transfection of v-*ras*^{H} DNA into MCF-7 Human Breast Cancer Cells Bypasses Dependence on Estrogen for Tumorigenicity

Abstract. *The natural history of estrogen-responsive breast cancers often involves a phenotypic change to an estrogen-unresponsive, more aggressive tumor. The human breast cancer cell line, MCF-7, which requires estradiol for tumor formation in vivo and shows growth stimulation in response to estradiol in vitro, is a model for hormone-responsive tumors. The v-*ras^H *onc gene was transfected into MCF-7 cells. The cloned MCF-7$_{ras}$ transfectants, which expressed the v-*ras^H *messenger RNA and v-*ras^H *p21 protein (21,000 daltons), were characterized. In contrast to the parental cell line, MCF-7$_{ras}$ cells no longer responded to exogenous estrogen in culture and their growth was minimally inhibited by exogenous antiestrogens. When tested in the nude mouse, the MCF-7$_{ras}$ cells were fully tumorigenic in the absence of estrogen supplementation. Thus, cells acquiring an activated onc gene can bypass the hormonal regulatory signals that trigger the neoplastic growth of a human breast cancer cell line.*

The human breast adenocarcinoma cell line MCF-7 (*1*) is fully transformed according to the criteria of immortalized growth, lack of contact inhibition, characteristic changes in cellular morphology, and anchorage-independent proliferation in soft agar. Although these cells grow in culture without estrogens, they show accelerated growth in the presence of estrogens (*2*). This response to estrogen is mediated by hormone receptors and has been linked to the induction of several cellular products and secretory growth factors (*3*). Antiestrogens such as tamoxifen and LY 117018 specifically inhibit the proliferation of these cells (*4*).

When implanted in nude mice, MCF-7 cells are tumorigenic only in the presence of estrogenic hormones (*5*). We conducted experiments to find out if such a hormone-dependent phenotype could be abrogated by onc gene activation. As a model we selected the viral v-*ras*^{H} gene of Harvey murine sarcoma virus (Ha-MuSV) because it has known oncogenic potential (*6*) and has a detectable protein product of 21,000 daltons, p21, which is readily distinguishable from the cellular c-*ras*^{H} p21 protein because of Thr^{59} phosphorylation (*7*). The v-*ras*^{H} p21 protein differs from the normal human cellular homolog (c-*ras*$^{H-1}$) at only two amino acids, 12 and 59 (*7*).

A plasmid containing a complete permuted copy of the Ha-MuSV genome including three long terminal repeats (LTR's) in tandem (see top of Fig. 1) was transfected into MCF-7 cells in monolayer cultures (*8*) in the presence of the Eco-*gpt* selectable gene marker contained in the plasmid pSV2-*gpt* (*9*). In the *gpt*-selective medium, cells transfected with Eco-*gpt* alone (MCF-7$_{gpt}$) or Eco-*gpt* and Ha-MuSV DNA (MCF-7$_{ras}$) yielded 15 to 25 surviving colonies 14 days after transfection of 5×10^5 cells. The following enzyme phenotypes, polymorphic in the human population, were examined in the transfectants: lactic dehydrogenase, glucose-6-phosphate dehydrogenase, phosphoglucomutase-1, esterase-D, adenylate kinase, and glyoxylase-1. Allozyme typing revealed that MCF-7$_{gpt}$ and MCF-7$_{ras}$ did not differ significantly from the parental MCF-7 cells. Karyotype analysis showed that the MCF-7$_{ras}$ cells contained a specific chromosome marker described for MCF-7 cells (*1*).

We first confirmed the presence of Ha-MuSV DNA in MCF-7$_{ras}$ cells. Genomic DNA from the transfectants was examined by Southern blot analysis. Hybridization of Pst I-digested DNA to a v-*ras*^{H} probe showed two to three novel bands that were absent in the untransfected MCF-7 DNA (Fig. 1A), suggesting that multiple copies of v-*ras*^{H} sequences had been incorporated in tandem into the transfectants. The 4.4 kilobase (kb) Pst I fragment is similar in size to the Pst I fragment of the input cloned viral DNA. Hybridization of the Pst I (Fig. 1A) and Bam HI (Fig. 1B) digests indicated that the donor DNA had undergone complex rearrangements and deletions as shown by many hybridizing fragments of different sizes. The 4.4 and 3.3 kb Pst I bands in Fig. 1A probably represent fragments with three LTR's and one LTR, respectively. Instability of tandem LTR's has been noted previously (*10*). As expected, no exogenous sequences homologous to v-*ras*^{H} were detected in control MCF-7 (Fig. 1) or pSV2-*gpt* transfected MCF-7 cells.

Digestion of the transfected DNA with Xho I, an enzyme that does not cut the plasmid, revealed two *ras* bands at 14.7 and 20.5 kb in MCF-7$_{ras}$ but not in the wild-type MCF-7 DNA (Fig. 1C). Since the Xho I fragment sizes were greater than the original length of intact plasmid (10.7 kb), these results demonstrated that the plasmid DNA had been integrated into the host cell's DNA. This integration was stable and invariant during extended cell passage in culture in the absence of selective medium (Fig. 1D).

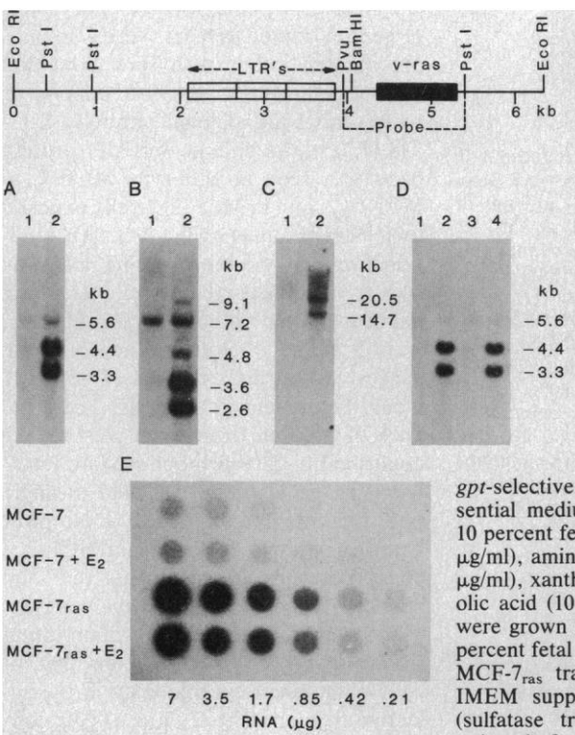

Fig. 1. Detection and expression of v-*ras*^{H} sequences in MCF-7 transfectants and map showing plasmid clone fragment generated by Eco RI digestion. (A to D) Genomic DNA (10 μg) isolated from wild-type MCF-7 cells (lanes 1 and 3) or MCF-7$_{ras}$ transfectants (lanes 2 and 4) was digested with (A and D) Pst I, (B) Bam HI, or (C) Xho I, and subjected to Southern analysis (*11*) by hybridization with a ^{32}P nick-translated Pvu I–Pst I fragment probe which contains the v-*ras*^{H} gene and some viral flanking sequences. (A, B, and C) MCF-7$_{ras}$ transfectants were grown in the *gpt*-selective medium: improved minimal essential medium (IMEM) supplemented with 10 percent fetal calf serum, hypoxanthine (15 μg/ml), aminopterin (2 μg/ml), thymidine (10 μg/ml), xanthine (250 μg/ml), and mycophenolic acid (10 μg/ml). Wild-type MCF-7 cells were grown in IMEM supplemented with 10 percent fetal calf serum. (D) MCF-7 cells and MCF-7$_{ras}$ transfectants were propagated in IMEM supplemented with 5 percent CCS (sulfatase treated calf serum, which was stripped of endogenous hormones by dextran coated charcoal treatment) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of $5 \times 10^{-9} M$ E_2. (E) Total cellular RNA was isolated from wild-type MCF-7 cells or MCF-7$_{ras}$ transfectants growing in the absence or presence of $5 \times 10^{-9} M$ E_2. Samples were dotted directly onto the nitrocellulose paper and hybridized with ^{32}P-labeled v-*ras*^{H} probe.

Table 1. Total cellular E_2 and progesterone specific receptor concentrations. Whole cell equilibrium binding assays were performed as described previously (*16*); K_d, dissociation constant. Data are the average of duplicate determinations (variability is less than 20 percent).

Cells	Estradiol receptor		Progesterone receptor (sites per cells)	
	Sites per cell	K_d (*M*)	Without E_2	With E_2
MCF-7	75,737	1.76×10^{-10}	70,652	115,992
MCF-7_{gpt}	78,913	2.39×10^{-10}	43,860	74,971
MCF-7_{ras}	45,795	3.42×10^{-10}	28,636	118,480

Further, there was no change in the intensities of the hybridizing bands when the cells were grown in medium supplemented with estradiol (E_2) (Fig. 1D).

Total cellular RNA was prepared from untransfected and transfected MCF-7 cells and dot blot analysis was performed to quantitate the levels of v-*ras*^{H} transcripts. Densitometric scans of the blots showed that the transfectants expressed *ras*^{H} RNA at levels 10 to 12 times higher than wild-type MCF-7 cells (Fig. 1E) or contained a low level of endogenous c-*ras*^{H} RNA and neither its expression nor that of the exogenously acquired v-*ras*^{H} gene was altered by E_2 treatment (Fig. 1E).

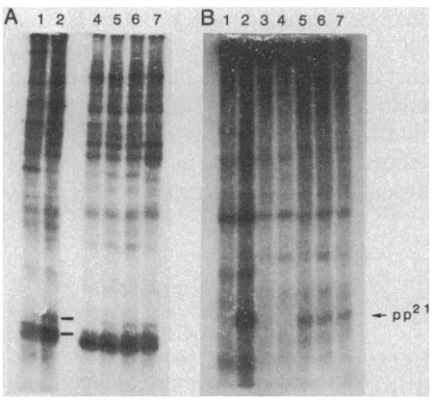

Fig. 2. The presence of v-*ras*^{H} related p21 in MCF-7_{ras} transfectants. Cultures were incubated with [^{35}S]methionine (200 μCi/ml) or [^{32}P]orthophosphate (300 μCi/ml) at 37°C for 16 hours. Cells were lysed in buffer containing 10 m*M* sodium phosphate (*p*H 8.0) 1 m*M* EDTA, 100 m*M* NaCl, 1 percent Triton X-100, 0.5 percent sodium deoxycholate, 0.1 percent sodium dodecyl sulfate (SDS), and 2 m*M* phenylmethylsulfonyl fluoride. The supernatant was clarified at 100,000*g* for 30 minutes at 4°C prior to immunoprecipitation. Normal rat serum was substituted for antibody in control precipitations. The immune complex was subjected to SDS-polyacrylamide gel electrophoresis on 15 percent polyacrylamide gels as described (*14*). (A) [^{35}S]Methionine-labeled extracts. (B) [^{32}P]Orthophosphate-labeled extracts. Cell extracts were prepared from NIH 3T3 cells (lane 1), NIH 3T3 cells transformed with Ha-MuSV (lane 2), wild-type MCF-7 cells (lane 3), MCF-7_{gpt} transfectants (lane 4), MCF-7_{ras} transfectants (lane 5), MCF-7_{ras} transfectants propagated in the absence of selective medium (lane 6), and secondary cultures derived from tumors induced with MCF-7_{ras} cells in nude mice (lane 7). The two lines between lanes 2 and 4 in panel A indicate the positions of pp21 (upper line) and p21 (lower line).

We also investigated the production of the v-*ras*^{H} p21 in MCF-7_{ras} cells. This protein, which is associated with the plasma membrane and binds guanine nucleotides (*7, 12*), has a phosphoacceptor threonine at residue 59, leading to phosphorylation of about one-third of the viral p21 in rodent cells. The viral p21 therefore migrates as a doublet composed of a more slowly migrating phosphorylated form (pp21) and a more rapidly migrating nonphosphorylated form of the protein (p21). By contrast, cellular p21 is not phosphorylated because it lacks the threonine residue at position 59; its p21 is detected as a single band. Cells were incubated with [^{35}S]methionine to label p21 and pp21 and with [^{32}P]orthophosphate to label pp21. Detergent-extracted lysates were immunoprecipitated either with sera from rats bearing Ha-MuSV induced tumors or with v-*ras*^{H} monoclonal antibody Y13-238 (*7, 13*). An endogenous p21 protein band was seen in wild-type MCF-7, in MCF-7_{gpt}, and in MCF-7_{ras} cells exposed to [^{35}S]methionine (Fig. 2A). An additional upper band representing pp21 was seen in v-*ras*^{H} transfected cells after long exposure of the autoradiogram (Fig. 2A, lanes 5, 6, and 7), although the RNA data suggested that higher levels of viral p21 would be present in the cells. The presence of pp21 in these cells was further confirmed by [^{32}P]orthophosphate labeling (Fig. 2B), which revealed a single pp21 band unique to MCF-7_{ras}. No pp21 band was seen in MCF-7 or in MCF-7_{gpt} cells. When *ras* transfectants that had been grown without selection were assayed for viral p21, the phosphorylation profile was not different from that of cells maintained continuously in the selective medium (Fig. 2, lane 6). We conclude that v-*ras*^{H} in MCF-7_{ras} is expressed as RNA and protein.

We wished to determine whether MCF-7_{ras} cells had an altered response to estrogen and antiestrogens. Since the effects of hormones are mediated by hormone receptors (*15*), we first assayed the amount of estrogen receptor in the MCF-7_{ras} transfectants, the control MCF-7_{gpt} cells, and wild-type MCF-7 cells (Table 1). Initially, we found that the steady-state level of E_2 receptor in these cells varied by no more than a factor of two. Furthermore, E_2-inducible progesterone receptor synthesis was equivalent in the control and MCF-7_{ras} transfectants indicating that the MCF-7_{ras} cells retained the mechanism for E_2 response. We next examined growth response to hormones, both by tritiated thymidine incorporation and by growth rate determination.

Since E_2 receptor content in MCF-7 cells is not constant, but undergoes autoregulation according to the proliferative state of the cells (*16*), isoleucine deprivation was used to synchronize the cells prior to measuring [^{3}H]thymidine incorporation into DNA in hormone treated and untreated cells (Fig. 3). Isoleucine deprivation for 30 hours in vitro led to proliferative arrest and a dramatic decline in [^{3}H]thymidine incorporation. In both wild-type MCF-7 and MCF-7_{gpt} cells, E_2 caused an almost twofold increase in the rate of thymidine incorpo-

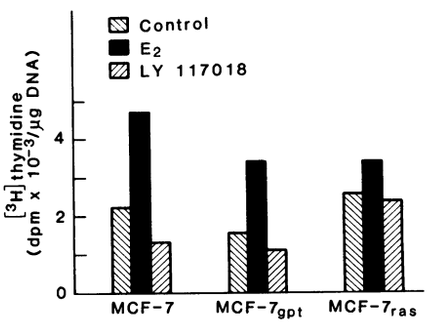

Fig. 3. Effects of estrogen and antiestrogen on [^{3}H]thymidine incorporation in growth-arrested cells. Wild-type and transfected MCF-7 cells (2×10^5) were plated in replicate in IMEM supplemented with 5 percent CCS. After 48 hours the medium was changed for isoleucine-depleted medium. After another 30 hours cells were rescued from growth arrest by changing the medium to isoleucine-supplemented medium in the absence or presence of 5×10^{-9}*M* E_2 or 10^{-8}*M* LY 117018 (6-hydroxy-2-hydroxyphenyl)-benzo(b)thien-3-yl-*p*-(2-(pyrrolidinyl) ethoxy phenyl ketone). Cells were exposed for 1 hour before being harvested to [^{3}H-methyl]thymidine (1 μCi/ml; 46 Ci/mmol) for 18 hours after the reversal of metabolic block. The cells were washed and harvested, and the radioactivity precipitable in 10 percent trichloroacetic acid was determined. Fluorometric determinations of DNA were also made (*17*). Data are the average of duplicate determinations. Variability was less than 15 percent.

ration into DNA at 18 hours after the reversal of the isoleucine block; conversely, the antiestrogen LY 117018 produced substantial inhibition of thymidine incorporation in these cells. In contrast, MCF-7_{ras} cells were minimally affected by E_2 and were comparatively insensitive to antiestrogen treatment.

As shown in Fig. 4, a physiologic concentration of E_2 stimulated, whereas, the LY 117018 strongly inhibited, growth of wild-type MCF-7 cells. E_2 was effective at concentrations as low as $10^{-10}M$. In contrast, MCF-7_{ras} cells demonstrated no augmented growth response to E_2. Equally striking was the abrogation of the growth inhibition induced by LY 117018. Similar results were also obtained with $10^{-6}M$ tamoxifen. Thus, MCF-7 cells with the v-*ras*^{H} gene were able to bypass much of the hormone responsive growth regulatory mechanism.

When female ovariectomized nude mice were inoculated with the control cells (MCF-7 and MCF-7_{gpt}), solid tumors developed after 3 to 4 weeks only if an E_2 supplement was given at the time of cell inoculation (Table 2). In the absence of estrogen, tumors did not develop even when the cell inoculum was increased five to ten times and the observation period was prolonged for 90 days. In contrast, MCF-7_{ras} cells produced tumors in the absence or presence of an E_2 source. Tumors from MCF-7_{ras} appeared as early as 2 weeks after inoculation and grew steadily. Thus, v-*ras*^{H} conferred hormone-independence to the tumorigenicity of MCF-7 cells in vivo.

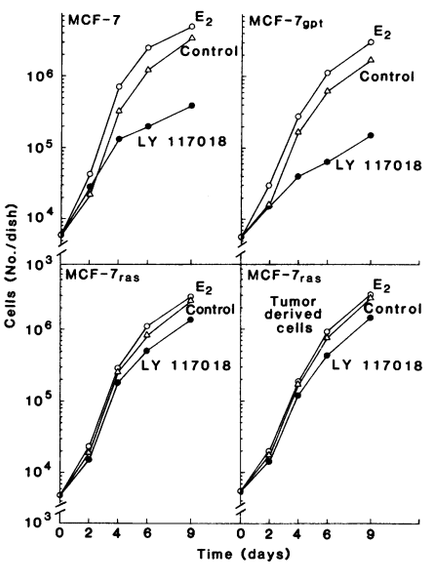

Fig. 4. Effects of E_2 and antiestrogen on cell proliferation of wild-type and transfected MCF-7 cells. Cells (approximately 2×10^4 per dish) were plated in replicate in IMEM supplemented with 5 percent CCS. Hormonal supplements (E_2, $10^{-9}M$; LY 117018, $10^{-8}M$) were added the next day. Cells were fed every 2 to 3 days and cells were counted in parallel dishes. Each point represents the mean of three determinations. Tumor-derived cells in culture were obtained from tumors induced with MCF-7_{ras} transfectants. The variation between triplicates was less than 15 percent.

Table 2. Tumor growth in the nude mice. Oophorectomized athymic nude mice (5 to 6 weeks old) with or without E_2 pellets (0.5 mg) were inoculated with 2×10^6 to 5×10^6 tumor cells subcutaneously into the area of the first mammary fat pad and observed for the formation of tumors. Results given are the sum of five different inoculations done on separate days. There was no statistically significant experiment to experiment variation.

Cells	Tumors formed/mice inoculated	
	Without E_2	With E_2
MCF-7	0/20	8/12
MCF-7_{gpt}	0/10	6/8
MCF-7_{ras}	26/32	10/12

Explants of some representative tumors were placed in tissue culture and analyzed for the presence of v-*ras*^{H} related sequences. Southern blots (Fig. 5) showed v-*ras*^{H} related fragments identical in size to the parental MCF-7_{ras} transfectants. Cells derived from a hormone-dependent tumor induced with pSV2-*gpt* transfected MCF-7 cells did not reveal v-*ras*^{H} related sequences. These results confirm the expansion of the same clonal population of tumorigenic cells without additional alteration of the v-*ras*^{H} sequence organization within the tumor cells. Viral p21 could be immunoprecipitated from the secondary culture derived from MCF-7_{ras} induced tumors in nude mice (Fig. 2, lanes 7). The tumor-derived cells in culture behaved similarly to the v-*ras* transfectants in continous culture in their minimal responsiveness to E_2 and antiestrogens (Fig. 4).

Our data show that a potentially tumorigenic human cell line transfected with an exogenous activated onc gene can bypass a hormonal mechanism that customarily triggers tumor formation by that cell. The activated *ras* gene product itself is mitogenic in a variety of rodent cells (*18*). This gene product either by itself or by interaction with cellular regulatory molecules evidently induces autonomous growth in hormone-dependent MCF-7 cells. Since the transfected cells were not inhibited by antiestrogens and since their hormone responsiveness as measured by the E_2 receptor content and induction of progesterone receptor was not markedly different from the parental cell line, it appears that the growth signals triggered by receptor-ligand interactions in MCF-7 cells are no longer recognized in the MCF-7_{ras} cells.

The relevance of cellular *ras* gene activation to human breast cancer is not well understood. There are only two reports of *ras* gene abnormalities in human breast cancer cell lines. One group of investigators, using DNA transfection and extensive recipient cell passage in nude mice, has isolated, from MCF-7 DNA, three transforming genes (*19*). One of these genes is *ras*^{N}, which is amplified five to ten times. The other two genes are not homologous to known onc genes. C-*ras*^{H} activation has been noted in another hormone-independent breast cancer cell line, HS578T (*20*). We have not found any effect of estrogen stimulation on c-*ras*^{H}, *myc*, *mht*, *erb*^{B}, *sis*, or *myb* gene expression in MCF-7 cells.

Most studies of onc gene transfection have focused on rodent cells. Our model with a human cancer cell line provides one framework for explaining specific in vivo biological effects consistent with progression of human breast cancer. Frequently, the subset of human breast cancers that is E_2-responsive in the early stages evolves to become E_2-unresponsive, at which time it displays a more aggressive behavior. Our data suggest

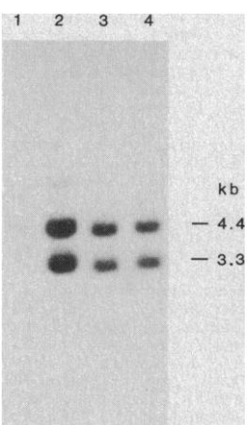

Fig. 5. Detection of v-*ras*^{H} sequences in secondary cultures derived from tumors induced with MCF-7_{ras} transfectants in nude mice. Genomic DNA (10 μg) was digested with Pst I, subjected to electrophoreses, filter-blotted, and hybridized to ^{32}P-labeled v-*ras*^{H} probe. Lane 1, cells derived from MCF-7_{gpt} E_2-induced tumor; lane 2, MCF-7_{ras} cells in continuous culture; lanes 3 and 4, cells derived from tumors induced with MCF-7_{ras} in the presence (lane 3) or absence (lane 4) of E_2 supplementation to the animals. Solid tumors formed from injection of MCF-7 transfectants into nude mice were excised, finely minced, and disrupted prior to selection for resistance in vitro to the *gpt*-selective medium.

that a newly activated oncogene could convert an E_2-responsive tumor cell to one that is hormonally unresponsive; this change could provide a selective growth advantage to the E_2-unresponsive cell and eventually result in the grossly apparent autonomy of the tumor phenotype. An analogous situation may have been described in a melanoma patient, where one metastatic tumor had an activated ras^N gene, but the other metastases did not (*21*). We do not propose that *ras* activation is the only mechanism by which a breast cancer might alter its hormone dependent phenotype. We conclude, however, that amplification or mutation in a single gene may radically alter the growth regulatory and tumorigenic behavior of a cell that already has malignant potential.

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Gene for Human Insulin Receptor: Localization to Site on Chromosome 19 Involved in Pre-B-Cell Leukemia

Abstract. *Consistent chromosomal translocations in neoplastic cells may alter the expression of proto-oncogenes that are located near the breakpoints. The complementary DNA sequence of the human insulin receptor is similar to those of the EGF receptor (*erbB *oncogene) and products of the* src *family of oncogenes. With in situ hybridization and Southern blot analysis of somatic cell hybrid DNA, the human insulin receptor gene was mapped to the distal short arm of chromosome 19 (bands p13.2→p13.3), a site involved in a nonrandom translocation in pre-B-cell acute leukemia.*

Insulin initiates its biological effects by binding to specific membrane receptors on target cells (*1*). The mature human insulin receptor is a transmembrane glycoprotein comprised of two α subunits (molecular weight of ~130,000) and two β subunits (molecular weight of ~90,000) (*2*). The α subunit contains the insulin binding site (*3*). The β subunit, bearing an adenosine triphosphate (ATP)–binding site, possesses a tyrosine protein kinase activity (*4*) that catalyzes both the autophosphorylation of the β subunit and the insulin-dependent phosphorylation of exogenous protein substrates (*5*). The α and β subunits of the mature insulin receptor are synthesized as a single polypeptide chain (*6*). A complementary DNA (cDNA) for the precursor of the human insulin receptor has been isolated and characterized (*7*). We have now mapped the insulin receptor gene (*INSR*) by in situ hybridization and Southern blot analysis of rodent × human somatic cell hybrid lines.

In situ hybridization to human chromosome preparations was carried out with a ^{3}H-labeled subclone (pHP/HIR 12.1) containing a 4.2-kilobase (kb) insert fragment that includes the entire β- and

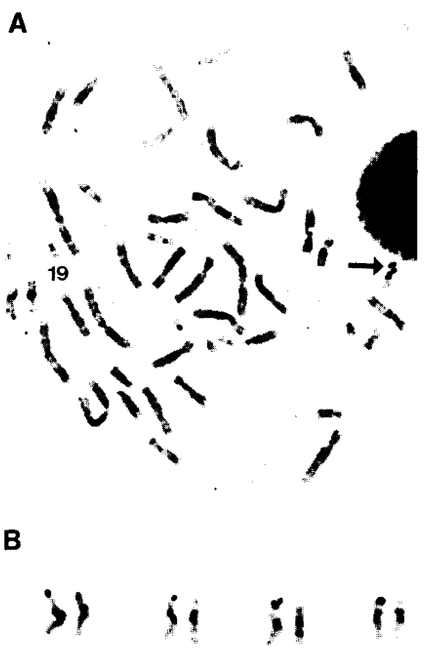

Fig. 1. G-banded human chromosomes after in situ hybridization with ^{3}H-labeled *INSR* probe. Human metaphase and prometaphase chromosomes were prepared from methotrexate-synchronized peripheral blood cell cultures (*25*) of two normal individuals. The pHP/HIR 12.1 was ^{3}H-labeled to a specific activity of 1.4×10^7 cpm/μg by nick-translation with [^{3}H]dCTP, [^{3}H]dATP, and [^{3}H]dTTP. In situ hybridization was carried out according to the method of Harper and Saunders (*26*). The probe was hybridized to chromosome preparations at a concentration of 25 ng/ml and 50 ng/ml for 15 hours at 37°C. The slides were exposed to Kodak NTB-2 emulsion for 10 to 14 days at 4°C. After autoradiographic development, the chromosomes were stained with quinacrine mustard dihydrochloride and photographed under a fluorescence microscope. The chromosomes were then G-banded with Wright's stain and a second photograph was taken of the previously selected cells. G-banded chromosomes were analyzed for silver grain localization. (A) Representative normal female metaphase spread with silver grains on the short arm of chromosome 19 (arrow) and low background labeling. The number 19 is directly below the unlabeled chromosome 19. (B) Pairs of chromosome 19 from 4 prometaphase cells, illustrating typical labeling at the distal short arm (bands p13.2→p13.3).