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Genetics

A tumor suppressor locus within 3p14-p12 mediates rapid cell death of renal cell carcinoma *in vivo*

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ABSTRACT **High frequency loss of alleles and cytogenetic aberrations on the short arm of chromosome 3 have been documented in renal cell carcinoma (RCC). Potentially, three distinct regions on 3p could encode tumor suppressor genes involved in the genesis of this cancer. We report that the introduction of a centric fragment of 3p, encompassing 3p14-q11, into a highly malignant RCC cell line resulted in a dramatic suppression of tumor growth in athymic nude mice. Another defined deletion hybrid contained the region 3p12-q24 of the introduced human chromosome and failed to suppress tumorigenicity. These data functionally define a tumor suppressor locus, nonpapillary renal carcinoma-1 (*NRC-1*), within 3p14-p12, the most proximal region of high frequency allele loss in sporadic RCC as well as the region containing the translocation breakpoint in familial RCC. Furthermore, we provide functional evidence that *NRC-1* controls the growth of RCC cells by inducing rapid cell death *in vivo*.**

Detailed cytogenetic investigation and loss of heterozygosity (LOH) studies have shown that the primary genetic aberration in human nonpapillary renal cell carcinoma (RCC) involves the short arm of human chromosome 3. In the rare familial form of the disease, defined translocations have been identified with breakpoints in the region of 3p13-p14 (1–3). High frequency LOH in sporadic RCC has been observed in two distinct regions on 3p encompassing 3p13-p14.3 and 3p21.3 (4–11). A third, more distal region mapping to 3p25 has been implicated in the etiology of Von Hippel–Lindau disease (12–14), an autosomal dominant disorder with many clinical manifestations including the development of bilateral kidney cysts. These cysts progress to RCC in some individuals (15, 16). Thus, three regions on 3p could be involved in the genesis of nonpapillary RCC.

Previous studies have shown that it is possible to complement the genetic defect in particular human cancers (that show high-frequency LOH on a specific chromosome) by the introduction of a normal copy of that chromosome containing a putative tumor suppressor gene (17–22). Using this approach, Shimizu *et al.* (21) modulated the tumorigenicity of the cell line YCR by the introduction of a human translocation chromosome containing 3p.

For our studies, we dissected 3p into defined regions using a functional assay to determine if a particular region alone could confer tumor suppression in RCC. We report the definition of a tumor suppressor locus within the most proximal region of high-frequency LOH in sporadic RCC as well as the region containing the translocation breakpoint in familial RCC.

MATERIALS AND METHODS

Cell Lines and Construction of Hybrids. The sporadic nonpapillary RCC cell line SN12C was derived from the primary kidney tumor of a 43-year-old man and carries a t(3;8) translocation with a breakpoint at 3p14 (23). A subcloned line (SN12C.19) of mixed granular/clear cell morphology was isolated and used for these studies. The microcell hybrid HA(3)BB containing human chromosomes 3 has been described (24). HA(3)IIaa contains a centric fragment of 3p (3p14-q11) in the A9 (mouse fibrosarcoma) background.

Microcell Fusion. The method of microcell fusion has been described (24, 25). Briefly, HA(3)BB cells were incubated in Colcemid at 0.06 μg/ml (48 hr) to induce micronucleation. Microcells were isolated after centrifugation of micronucleate cell populations in cytochalasin B (10 μg/ml) at 27,000 × *g* for 70 min (28–32°C). Microcells were resuspended in 4 ml of phytohemagglutinin P and added to a 70–80% confluent monolayer of RCC cells in each of two 25-cm^2 flasks. After a 15- to 20-min incubation (37°C) in phytohemagglutinin P, microcells were fused to RCC cells in 1 ml of 50% (wt/wt) polyethylene glycol (Koch Chemicals, England) for 1 min. Twenty-four hours postfusion, hybrid clones were selected by plating in medium containing G418 at 750 μg/ml and hypoxanthine/aminopterin/thymidine (A9 is hypoxanthine phosphoribosyltransferase negative). Each fusion produced between 87 and 134 G418-resistant clones.

Fluorescence *in Situ* Hybridization (FISH). Chromosome preparations were hybridized at 37°C with biotinylated human placental (100 ng), pSV2neo (400 ng), or D3S3 (400 ng) DNAs in 2× SSC/50% (vol/vol) formamide/10% dextran sulfate/sonicated salmon sperm DNA. After a wash in 50% formamide/2× SSC at 42°C for total human DNA and 37°C for pSV2neo and D3S3, the probe was detected with fluorescein-avidin and biotinylated anti-avidin antibody.

For chromosome painting, 400 ng of chromosome-specific DNA (26) and 50× C_0t 1 unlabeled DNA were hybridized as above to chromosome preparations. Posthybridization washing was performed at 42°C followed by 2× SSC at 37°C and detection as above.

***In Vivo* Growth Assays.** Microcell hybrid and parental RCC cells were injected subcutaneously at 5 × 10^6 cells into each of three 5- to 6-week-old male athymic nude mice (Harlan–Sprague–Dawley). Tumor volumes were monitored biweekly. Tumors were excised, explanted into culture, and maintained without G418 for chromosome analysis. For staging tumorigenicity assays, hybrid lines and parental controls were injected into each of six athymic nude mice.

RESULTS

Construction and Characterization of Monochromosomal Microcell Hybrid Clones. The monochromosomal hybrid HA(3)BB (24), which contains an intact, *neo*-marked human chromosome 3 in the A9 cell background, served as the donor line for microcell fusion into SN12C.19 (25, 27). Thirty of 87

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Abbreviations: LOH, loss of heterozygosity; RCC, renal cell carcinoma; FISH, fluorescence *in situ* hybridization.

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Table 1. Characterization of microcell hybrid clones

Cell line injected into nude mice	Chromosome 3 retention in RCC background
SN12C.19	—
SN19(3)D	Intact 3
SN19(3)CCC	Intact 3
SN19(3)VV	Intact 3
SN19(3)A	Intact 3 (pericentric inversion)
SN19(3)WW	Intact 3 (2 copies)
SN19(3)N	Del(3p13) t(3;11)
SN19(3)EEE	3p12-q24
SN19(3i)YY	3p14-q11
SN19(3i)FF	3p14-q11
SN19(3i)LL	3p14-q11

microcell hybrid clones were screened by FISH using chromosome 3-specific DNA as a probe to detect the presence of the introduced human chromosome. Seven hybrids were characterized further by FISH using pSV2neo as a probe and by G-banding to determine if the introduced chromosome had suffered any rearrangements upon entry into the recipient cell background. Karyotypic examination of microcell hybrid clones revealed that 3 of 7 SN19(3) series hybrids contained an intact, unrearranged copy of the introduced chromosome 3 (Table 1). One hybrid [SN19(3)EEE] contained large terminal deletions of 3p and 3q and retained only 3p12-q24 (Table 1).

Introduction of Human Chromosome 3 Results in RCC Tumor Suppression. Five of these SN19(3) series microcell hybrids and parental RCC cells were injected (at 5×10^6 cells) subcutaneously into each of three athymic nude mice. After 8 weeks, RCC cells formed tumors of an average wet weight of 0.9–1.0 g. In three separate experiments, the introduction of chromosome 3 into SN12C.19 resulted in dramatic tumor suppression in all hybrids containing an intact chromosome 3 (Fig. 1*a*). The only SN19(3) microcell hybrid that formed large tumors was SN19(3)EEE, which contained only 3p12-q24. These data provide functional evidence for a tumor suppressor locus on human chromosome 3 that must reside either distal to 3q24 or, more probably, distal to p12 on the short arm of human chromosome 3.

Identification and Characterization of Fragment-Containing Clones. To define the exact region containing the tumor suppressor gene on 3p, we analyzed a collection of A9 microcell clones generated previously (24) that had been screened with 52 chromosome 3-specific PCR primers and shown to contain 3p fragments (A.M.K. and S. L. Naylor, unpublished results). PCR (29) and Southern analyses (7, 30) were performed on these fragment-containing hybrids using primers to β-galactosidase (*GLB1*), which maps to 3p21-p22 (29), or D3S3 as a probe for 3p12-p14 (31). Any clones that were positive for D3S3 and negative for *GLB1* were further analyzed by FISH using total human DNA as a probe. One clone [HA(3)II] was identified that contained one to five 3p fragments per metaphase and of the size range of 15–20 megabases. Subcloning HA(3)II produced the hybrid clone HA(3)IIaa (Fig. 2*a*), which contains a single, centric fragment of 3p encompassing 3p14-q11. FISH using pSV2neo (Fig. 2*b*) and D3S3 as probes (Fig. 2*c*) confirmed the origin of the fragment and the location of the integration site near the centromere on 3q. As further evidence that the 3p fragment was not a discontinuous and rearranged fragment of chromosome 3, chromosome painting of normal human metaphase spreads was performed by inter-*Alu* PCR amplification of human DNA from HA(3)IIaa. The results indicated that the 3p fragment mapped back exclusively to the region 3p14-q11 (Fig. 2*d*). HA(3)IIaa was also screened by PCR using primers within the Von Hippel–Lindau disease (*VHL*) gene, which maps to 3p25 (32). Results indicated that the fragment in HA(3)IIaa did not contain the *VHL* gene (data not shown).

The 3p fragment was transferred into SN12C.19 via microcell fusion. FISH using a chromosome 3-specific centromeric repeat probe (D3Z1) (33), pSV2neo, and D3S3 was carried out to confirm retention of the 3p fragment in the human RCC cell background (data not shown). *In vitro* growth of hybrid clones was monitored in 10% serum, and population doubling times were determined after 8–12 days of logarithmic growth. Results indicated very similar doubling times for parental RCC cells (22 hr) and 4 SN(19)3 hybrids

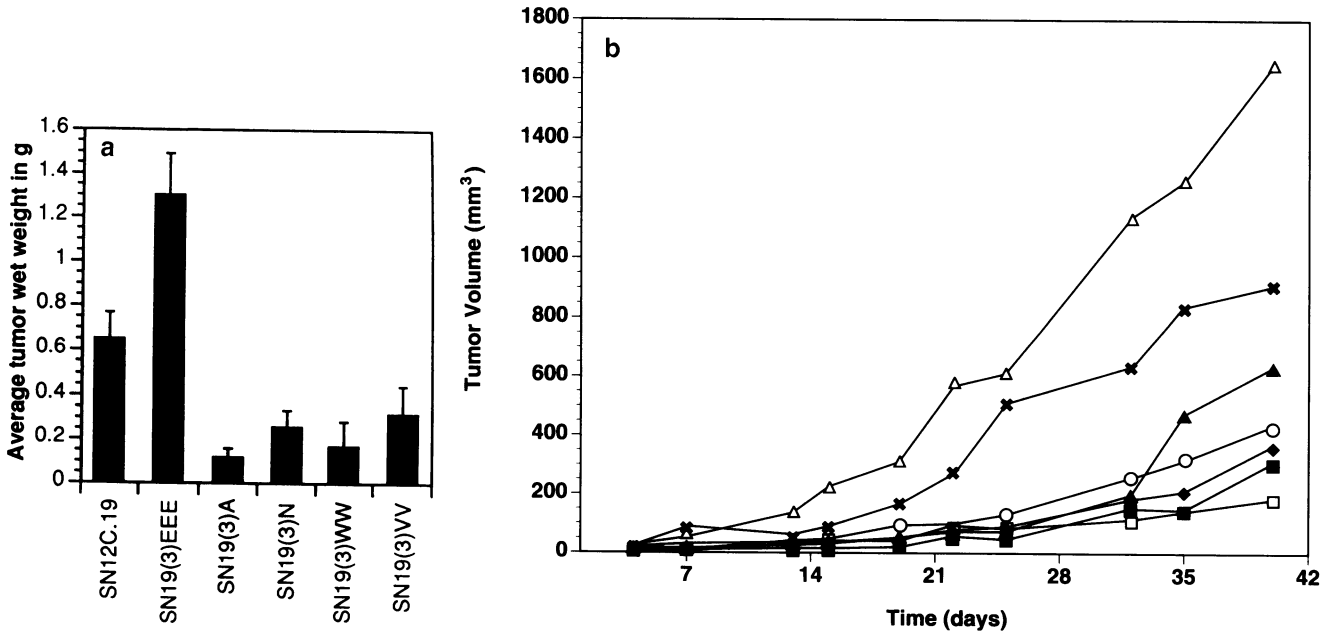

FIG. 1. Tumorigenicity assays. (*a*) Wet weights of tumors formed after injection of microcell hybrids and parental SN12C.19 cells into nude mice. (*b*) Average tumor volumes from staging tumorigenicity assay. Lines tested include SN19(3i)LL (▲), SN19(3i)FF (○), SN19(3i)YY (◆), SN19(3)WW (■), SN19(3)A (□), SN12C.19 (x), and SN19(3)EEE (△). Tumor volumes were calculated by the following formula: $[(W)^2 \times (L)]/2$ (28).

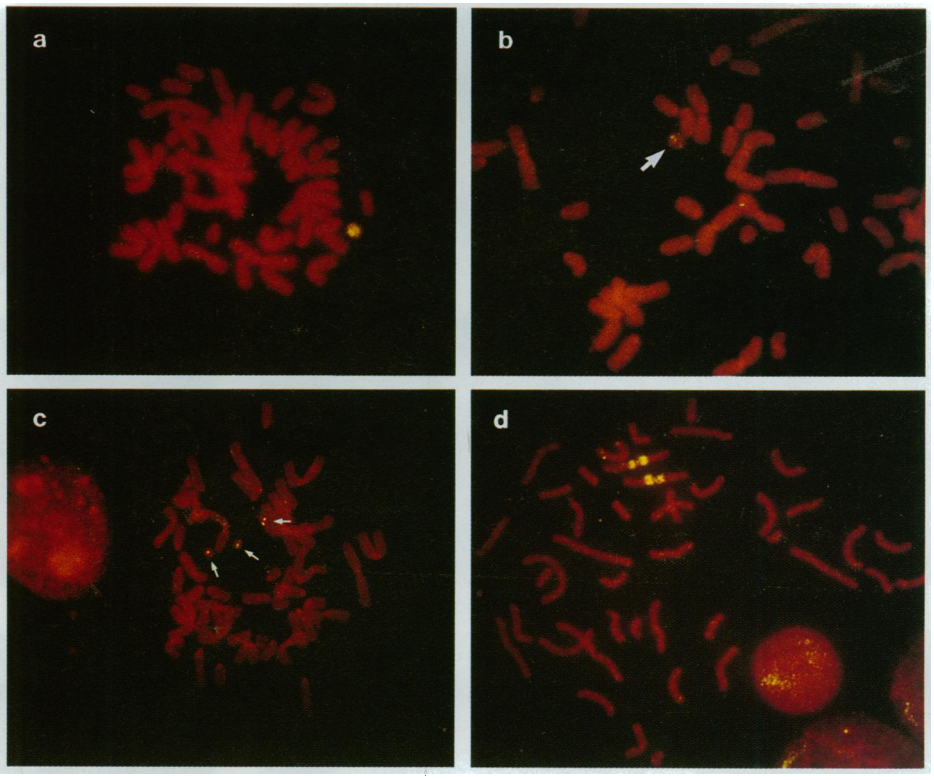

FIG. 2. FISH. (*a*) Total human DNA was used as a probe to detect the presence of the small 15- to 20-megabase fragment 3p14-q11 in HA(3)IIaa. (*b*) pSV2neo was used to detect *neo* in HA(3)IIaa. (*c*) D3S3 (3p14) was used to characterize fragments in HA(3)II prior to subcloning. Note three copies of the 3p fragment in the A9 cell. (*d*) Chromosome painting of a normal human metaphase spread using chromosome 3-specific DNA from HA(3)IIaa. The 3p fragment maps back exclusively to 3p14-q11.

containing chromosome 3 (average of 24 hr), as well as SN19(3)EEE, the control hybrid containing 3p12-q24 (26 hr), and SN19(3i)YY, the hybrid containing the introduced 3p14-q11 (27 hr).

Tumor Suppression Concomitant with Rapid Cell Death of RCC Mediated by the Introduction of Human Chromosome 3 or 3p Centric Fragment. For *in vivo* analyses, five SN19(3i) microcell hybrids were tested for tumorigenicity in athymic nude mice. In three different experiments, after 6 weeks *in vivo*, three 3p fragment-containing hybrids [SN19(3i)LL, SN19(3i)FF, and SN19(3i)YY] showed dramatic tumor suppression (Fig. 1*b*). Two of the clones failed to exhibit significant suppression (data not shown). Potentially, these clones could represent hybrids that segregated or rearranged the 3p fragment *in vivo*. To test this theory, the small tumors (formed following injection of chromosome 3 and fragment-containing clones into nude mice) were excised six weeks postinjection and histopathologically analyzed for mitotic index, necrosis, degree of invasiveness, vascularity, and cellular pleomorphism (34). In all cases, the small tumors derived from suppressed hybrid clones were found to be as highly malignant as the parental line SN12C.19 (data not shown). We reasoned that the hybrid cells must have been initially suppressed for growth *in vivo*, and at some time point, loss or inactivation of the tumor suppressor locus within this region resulted in tumor outgrowth. FISH experiments indicated that hybrid clones retained the centric fragment at high frequency (average of 93%) at the time of injection. At the end of the experiment, explants derived from small tumors in suppressed fragment-containing clones showed loss of the fragment with an average retention of 59%.

Histopathology was also performed on tumor tissue from a staging tumorigenicity assay. We wanted to determine if, at a given time point, segregation or inactivation of the suppressor locus from the injected cell population could be correlated with changes in tumor morphology and rapid expansion of the tumor cell population. Tumor volumes were determined biweekly, and tumors were excised weekly postinjection for histopathological analysis. Three fragment-containing clones and three hybrids containing an intact human chromosome 3 were injected subcutaneously (at 5×10^6 cells) into each of six athymic nude mice. SN19(3)EEE, which contains 3p12-q24, was injected along with the parental SN12C.19 cells.

At the end of the first week postinjection, the parental cell line and SN19(3)EEE displayed *in vivo* parameters of a mixed clear cell/granular cell nonpapillary carcinoma with high mitotic index, a high degree of invasiveness, and vascularization typical of RCC (Fig. 3, panel *1a*). However, the small tumors derived from the chromosome 3 and fragment-containing microcell hybrid clones contained a central core of dead or necrotic cells (Fig. 3, panels *1b* and *1c*). This high degree of necrosis is very unusual in such small tumors of 4–20 mm^3. Necrosis is usually associated with rapidly growing tumors of much larger size. Four of the six clones examined showed 60–70% necrosis. Two clones showed defined areas of necrosis but <60% cell death at the end of the first week [SN19(3)WW and SN19(3i)FF], and these were the most suppressed clones at the end of the first week *in vivo*. Slower *in vivo* growth might reflect such differences in the degree of necrosis at the end of the first week. Clearly, SN19(3i)FF was more necrotic at the end of the second week *in vivo*.

Rapid cell death *in vivo* was common to all suppressed fragment-containing clones and clones containing an intact chromosome 3. It was not apparent in parental cells or in control cells containing 3p12-q24. With time, tumors from the suppressed clones became vascularized (Fig. 3, panels *2b* and *2c*), similar to tumors from the parent and control clones (Fig.

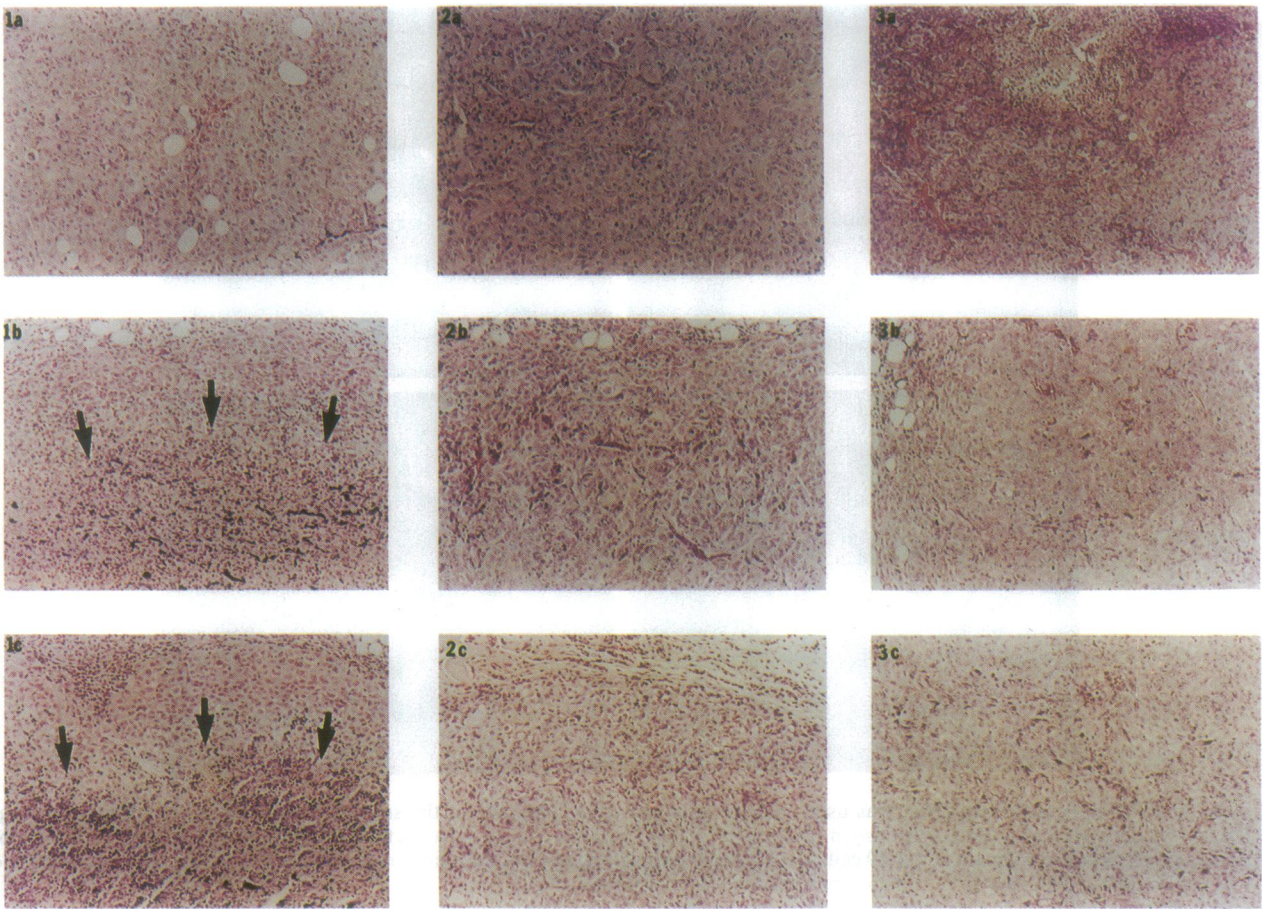

FIG. 3. Histopathology performed on tumors from the staging tumorigenicity assay. Clones tested include SN19(3)EEE, which contains 3p12-q24, at the end of week 1 (panel *1a*), week 2 (panel *2a*), and week 3 (panel *3a*) *in vivo*; SN19(3)A, the clone containing an intact chromosome 3, at the end of week 1 (panel *1b*), week 2 (panel *2b*), and week 3 (panel *3b*); SN19(3i)LL, the hybrid clone containing only the region 3p14-q11, at week 1 (panel *1c*), week 2 (panel *2c*), and week 3 (panel *3c*). Note the high degree of cell death and/or necrosis evident at the end of week 1 in the SN19(3)A and SN19(3i)LL (denoted by arrows). Cell death is not at all evident in the control clone containing 3p12-q24.

3, panel *2b*), with no significant necrosis. At the end of the experiment, tumor cells from the suppressed clones were highly aggressive by histopathological criteria (Fig. 3, panels *3b* and *3c*) and resembled tumors from the parent cell line and control clone (Fig. 3, panel *1a*). The parental RCC cell line and SN19(3)EEE were highly necrotic at the end of the experiment, as might be expected for large, rapidly dividing tumor cell populations.

DISCUSSION

Thus, introduction of the centric fragment encompassing 3p14-q11 into SN12C.19 resulted in dramatic tumor suppression resulting from rapid cell death following injection of cells into nude mice. Hybrids containing 3p12-q24 not only formed tumors equivalent to or larger than those formed by parental RCC cells but also morphologically resembled the parent line with no evidence of early cell death. These data define a genetic locus in the most proximal region of high frequency LOH in sporadic RCC as well as the region containing the translocation breakpoint in familial RCC (Fig. 4). We have designated the locus nonpapillary renal carcinoma 1 (*NRC-1*). Furthermore, by a concerted genetic and pathological analysis, we have shown a function for *NRC-1* prior to isolation of the gene. The function of *NRC-1* correlates directly with the induction of rapid cell death *in vivo*. The cell death observed does not seem to be a random rejection event as described by Stanbridge and Ceredig (35) on day 1 following injection of both tumorigenic and nontumorigenic HeLa × fibroblast hybrids into nude mice. Both tumorigenic and nontumorigenic hybrids exhibited cell death on day 1 followed by differentiation of the nontumorigenic cells by day 4. In sharp contrast to these results, in our studies all suppressed fragment-containing clones, chromosome 3-containing clones, the control tumorigenic hybrid SN19(3)EEE, and parental cells had very similar doubling times *in vitro*, yet *in vivo*, rapid cell death was only observed in the hybrids containing the intact chromosome 3 and the region 3p14-q11. Neither the parental RCC cells nor the control line SN19(3)EEE showed a central core of dying cells with no evidence of differentiation in any of the clones. In the suppressed clones, cells surrounding the core were viable most probably because of access to growth factors in the periphery of the tumor, which could explain how cells containing the tumor suppressor locus could escape growth control *in vivo*. Clearly, loss of the centric fragment from the tumor population at the end of the experiment was evident in some but not all of the cells in the tumor explants. Thus, *NRC-1* could function to mediate growth factor dependence in the RCC cells *in vivo*. *In vitro* experiments have confirmed a striking effect of the transferred fragment on the ability of RCC cells to proliferate in reduced serum concentrations (Y.S. and A.M.K., unpublished results).

To our knowledge, these experiments are the first report of the direct transfer of a centric fragment in a functional tumor suppression assay. Transfer of this small region of 3p was made possible by simple screening of A9 hybrid clones after microcell fusion. These results indicate the utility of this

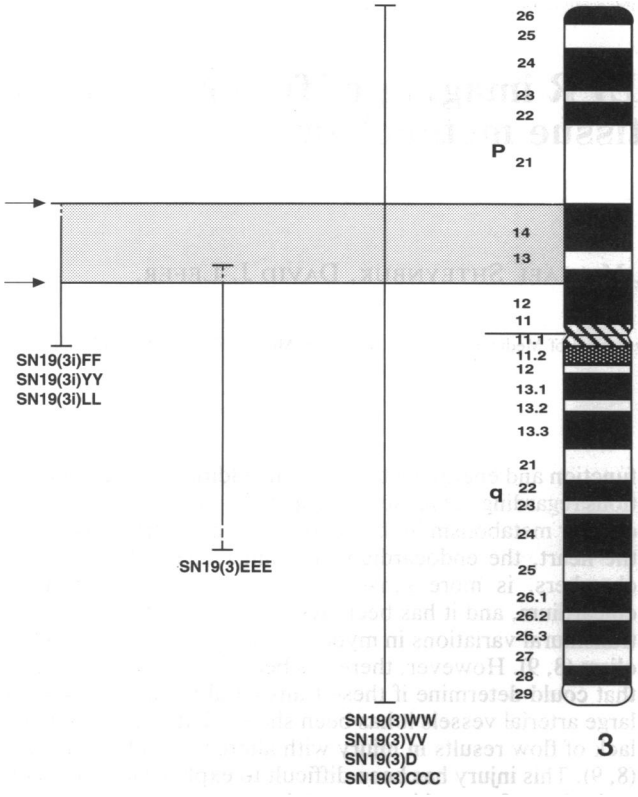

FIG. 4. Regional assignment of *NRC-1*. Depicted are suppressed hybrids containing an intact chromosome 3 as well as clones carrying 3p14-q11 in an RCC background; one hybrid is illustrated that was not suppressed *in vivo* and contained 3p12-q24. The region of nonoverlap containing *NRC-1* is denoted by arrows. Vertical dashed lines correspond to the breakpoint regions of fragment-containing clones.

system for the isolation of fragments surrounding the *neo* integration site in a tagged human chromosome. Furthermore, if the *neo* integration site is located close to the centromere, unrearranged centric fragments can be obtained. A potential caveat for these types of functional studies is the definitive proof of complementation. For our studies, the RCC recipient cell line contains a t(3;8) with a breakpoint in 3p14; however, without access to normal tissue from this patient, it is difficult to determine in this established cell line informative homozygous loss or inactivation at a particular locus. Nevertheless, this type of functional assay system in concert with molecular genetic and cytogenetic analyses of hybrid clones is a powerful approach for the definition of tumor suppressor genes.

Results from this study indicate that the proximal region of 3p is intimately involved in sporadic RCC. The interaction of this locus with more distal loci, such as the *VHL* gene, in the development of RCC remains to be determined. Recently, the *VHL* gene was isolated, and mutations were detected in VHL patients and in sporadic RCC cell lines (32). The finding that *NRC-1* functions to induce cell death *in vivo* provides strong evidence that this locus is involved in the genesis of nonpapillary RCC and potentially could play an important role in Von Hippel–Lindau disease-associated RCC as well.

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