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Resistance of human squamous carcinoma cells to transforming growth factor β1 is a recessive trait

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ABSTRACT **Because most human squamous carcinoma cell lines of the aerodigestive and genital tracts are refractory to the antiproliferative action of transforming growth factor β1 (TGFβ1) *in vitro*, we have begun to identify the causes for resistance of squamous carcinoma cell lines to TGFβ1 by using somatic cell genetics. Two stable hybrid cell lines (FaDu–HKc.1 and FaDu–HKc.2) were obtained by fusing a TGFβ1-resistant human squamous carcinoma cell line, FaDu-Hyg^R, with a human papilloma virus 16-immortalized, TGFβ1-sensitive, human foreskin keratinocyte cell line, HKc-neo^R. Whereas TGFβ1 did not inhibit DNA synthesis in parental FaDu-Hyg^R cells, it reduced DNA synthetic activity of HKc-neo^R, FaDu–HKc.1, and FaDu–HKc.2 cells by 75–85% (IC_{50}, 2–5 pM). Although squamous carcinoma cells express lower than normal levels of TGFβ1 type II receptors on their cell surface, TGFβ1 type II receptor mRNA was detected in all four cell lines. Recessive genes involved in TGFβ1 signaling may be localized to the distal portion of chromosome 18q, as this was the sole chromosomal region of homozygous deletion in parental FaDu-Hyg^R cells. Furthermore, our previous observation that mutant p53 decreases sensitivity of keratinocytes to TGFβ1 was supported by the finding that the level of the mutant p53 protein expressed by the hybrid cell lines was greatly reduced. In summary, TGFβ1 resistance of FaDu cells appears to be recessive and is presumably due to the loss of one or more post-receptor elements of the signaling pathway.**

The transforming growth factor β (TGFβ) family of 25-kDa polypeptides are potent inhibitors of epithelial cell growth (1). Roberts *et al.* (2) have proposed that the acquisition of cellular resistance to growth inhibitors such as TGFβ1 represents an important step in tumor development. This notion is supported by the observation that most epithelial carcinomas as well as B- and T-cell malignancies are refractory to growth inhibition by TGFβ1, whereas the proliferation of primary epithelial cells and of untransformed B and T lymphocytes is exquisitely sensitive to inhibition by TGFβ1 (see ref. 3 for review).

Human and murine epidermal keratinocytes secrete TGFβ1, which appears to act as an autocrine inhibitor of growth (4–7). TGFβ1 inhibits cell division by causing cells to arrest at the transition of G_1 to S phase and stimulates keratinocyte differentiation (5, 8, 9). The acquisition of resistance to TGFβ1 appears to represent an important step in the genesis of squamous-cell carcinoma (SqCC), as only transformed keratinocytes that have escaped from the negative control by TGFβ1 appear to give rise to invasive carcinomas (10, 11). Furthermore, most human SqCC lines are refractory to the antiproliferative activity of TGFβ1 (12).

To begin to identify the genetic cause(s) of TGFβ1 resistance and, thereby, the post-receptor elements in the TGFβ1 signaling pathway, we wished to determine whether the resistant phenotype was dominant or recessive by analyzing somatic cell hybrids of TGFβ1-sensitive human keratinocytes with TGFβ1-resistant SqCC cells.

MATERIALS AND METHODS

Cell Culture. The nontumorigenic, human papillomavirus type 16 (HPV16)-immortalized keratinocyte cell line HKc/HPV16d-R12 was obtained from Lucia Pirisi (University of South Carolina) (13, 14). A clonal, G418-resistant subline, HKc-neo^R, was used in the experiments described in this paper.

The human SqCC line FaDu, derived from a hypopharyngeal carcinoma (15), is completely refractory to inhibition of DNA synthesis by TGFβ1 (12, 16). A hygromycin B-resistant subclone of FaDu cells (FaDu-Hyg^R) was generated by infection with an amphotropic retroviral vector, pRVY-1, in which the gene that confers resistance to hygromycin B is expressed under control of a Moloney murine leukemia virus long terminal repeat (courtesy of David J. Riese and Daniel DiMaio, Department of Genetics, Yale University), followed by continuous selection in medium with hygromycin B at 160 μg/ml.

All cell lines were adapted to and maintained in serum-free MCDB153-LB++ medium, as described (12, 13).

Somatic Cell Hybridization. Somatic cell hybridization was performed as described (17, 18), with minor modifications. TGFβ1-sensitive HKc-neo^R cells were fused with TGFβ1-resistant FaDu-Hyg^R cells. In 60-mm tissue culture dishes, we plated 1×10^6 hygromycin B-sensitive HKc-neo^R cells together with 1×10^6 G418-sensitive FaDu-Hyg^R cells. Control dishes contained either 2×10^6 HKc/neo^R or 2×10^6 FaDu-Hyg^R cells only. After incubation at 37°C overnight, cells were exposed to 3 ml of 50% (wt/vol) polyethylene glycol (PEG-1000; J. T. Baker) in complete MCDB153-LB++ medium for exactly 1 min at room temperature. Twenty-four hours later, cells were replated at 0.8×10^6 per 100-mm dish in medium containing both hygromycin B (160 μg/ml) and G418 (150 μg/ml) in order to ensure outgrowth of true hybrids only. Individual colonies were ring-cloned and expanded in culture to allow further analysis.

Cytogenetic Analysis. Exponentially growing cells were treated with Colcemid (0.03 ng/ml) for 3–5 hr at 37°C prior to harvest. Cells were then subjected to hypotonic shock for 14–16 min with prewarmed 0.06 M KCl, which was mixed 1:1 with 0.6% (wt/vol) sodium citrate before use. Slides were baked at 90°C for 15–20 min before banding with trypsin and Giemsa stain. Fifteen metaphases of each cell line were photographed and fully karyotyped. Fluorescence *in situ*

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Abbreviations: TGFβ, transforming growth factor β; TβR, TGFβ receptor; HPV, human papillomavirus; SqCC, squamous-cell carcinoma.

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hybridization of metaphase chromosomes was performed with a "painting" probe specific for the whole human chromosome 18 (Oncor, Gaithersburg, MD), as recommended by the manufacturer (19).

Expression of TGFβ1 type II receptor (TβR-II) mRNA. The expression of TβR-II RNA in the four cell lines was quantified by RNase protection as described (20), with minor modifications. A cDNA probe for TβR-II (pH23FF) was obtained from R. A. Weinberg and H. Lin (Whitehead Institute, Cambridge, MA). The 5′ end (bp 35–596) of the TβR-II cDNA was subcloned into pBluescript II KS(+) (Stratagene). A *Pst* I digest of the resulting plasmid, pTGFβ1-5′, was used to generate a 440-nt (386 nt of plasmid sequence plus a 54-nt polylinker vector sequence) [α-^{32}P]dCTP (Dupont/NEN)-labeled antisense RNA probe with the Riboprobe Gemini II core system (Promega).

Total cellular RNA was extracted from confluent cells by the acid guanidinium thiocyanate–phenol/chloroform method (21). One hundred micrograms of total cellular RNA was hybridized to 20,000 cpm of gel-purified probe overnight at 42°C in 1× hybridization buffer, and the reaction mix was treated with RNase T1 (Boehringer Mannheim). Protected fragments were resolved in a 5% polyacrylamide/7 M urea gel and visualized by autoradiography at −80°C, using Kodak XAR film with intensifying screens.

Amplification and Sequencing of p53 Gene Segments. First-strand cDNA was synthesized from 10 μg of cellular RNA by reverse transcription using Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) and random hexamers as primers (22). Exon 7 (which contains codon 248) of the p53 gene was amplified from cDNA by the polymerase chain reaction (PCR) as described (16). We were able to distinguish by size the origin (DNA or RNA) of the PCR products by using two different sense PCR primers, one of which corresponded to the 3′ end of exon 6 (5′-TGGTGCCCTATGAGCCGCC-3′), while the other corresponded to the 5′ end of exon 7 (5′-TTGGCTCTGACTGTACCACCA-3′), and the same antisense primer corresponding to the 3′ end of exon 7 (5′-TGGAGTCTTCCAGTGTGA-3′). Sequencing of purified PCR products was performed as described (16).

RESULTS AND DISCUSSION

The frequent loss of responsiveness of malignant cells to the antiproliferative effect of TGFβ1 strongly suggests that resistance to TGFβ1 provides tumor cells with a selective advantage which, in turn, contributes to the malignant phenotype (3). An earlier study (12) showed that each of eight human SqCC lines of the aerodigestive tract and anogenital region was completely refractory to inhibition of DNA synthesis by TGFβ1. A comparison of the ^{125}I-TGFβ1-binding characteristics of these cells with those of non-neoplastic keratinocytes did not reveal any significant differences, except for an apparent reduction of the level of expression of TβR-II (12). These observations suggested that the resistant phenotype of the SqCC cells was either due to the loss of function of TβRs or due to the breakdown of post-receptor elements of the signal transduction pathway.

As the biochemical pathway by which TGFβ1's antiproliferative signal is transduced remains essentially unknown, we decided to use somatic cell genetics to begin to identify the mechanism(s) of resistance to TGFβ1. After fusion of TGFβ1-resistant, hygromycin-resistant FaDu SqCC cells with TGFβ1-sensitive, G418-resistant HKc-neo^R keratinocytes, the resulting hybrids were plated at low density and selected in the presence of both hygromycin and G418 (17, 18). Although numerous double-drug-resistant hybrids (23 colonies per 1000 cells plated) grew out initially, the vast majority underwent senescence, presumably because the two immortal parental cell lines belonged to different complementation groups with respect to lifespan genes (23). However, we eventually obtained two clonal hybrid cell lines (FaDu–HKc.1 and FaDu–HKc.2) which have now been maintained in culture for more than 50 and 54 passages, respectively. The morphology of both of these hybrid lines was intermediate between that of the two parental cell lines (Fig. 1). Cytogenetic analysis demonstrated that these cell lines were, in fact, true hybrids. The modal chromosome numbers of the parental lines were 80 and 55 for HKc-neo^R and FaDu-Hyg^R, respectively. Both hybrid lines were aneuploid, in the tetraploid to pentaploid range (92–115 chromosomes). FaDu–HKc.1 and FaDu–HKc.2 had a modal number of 124 and 103 chromosomes, respectively (Fig. 2).

TGFβ1 inhibited incorporation of [^{3}H]thymidine into DNA of exponentially growing HKc-neo^R, FaDu–HKc.1, and FaDu–HKc.2 cells by 75–85% (IC_{50}; 2–5 pM), whereas FaDu-Hyg^R cells remained unaffected (Fig. 3). Similarly, TGFβ1 did not affect growth of FaDu-Hyg^R cells in culture,

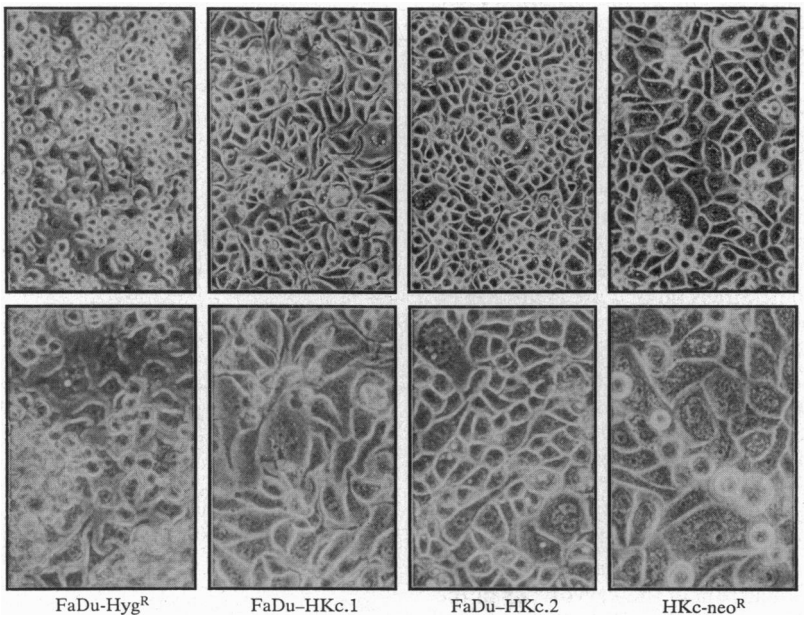

FIG. 1. Morphology of parental and somatic cell hybrid cell lines. Phase-contrast photomicrographs of confluent cultures were obtained by using a Nikon Diaphot inverted microscope and a Nikon F3 camera body. (*Upper*, ×375; *Lower*, ×750.)

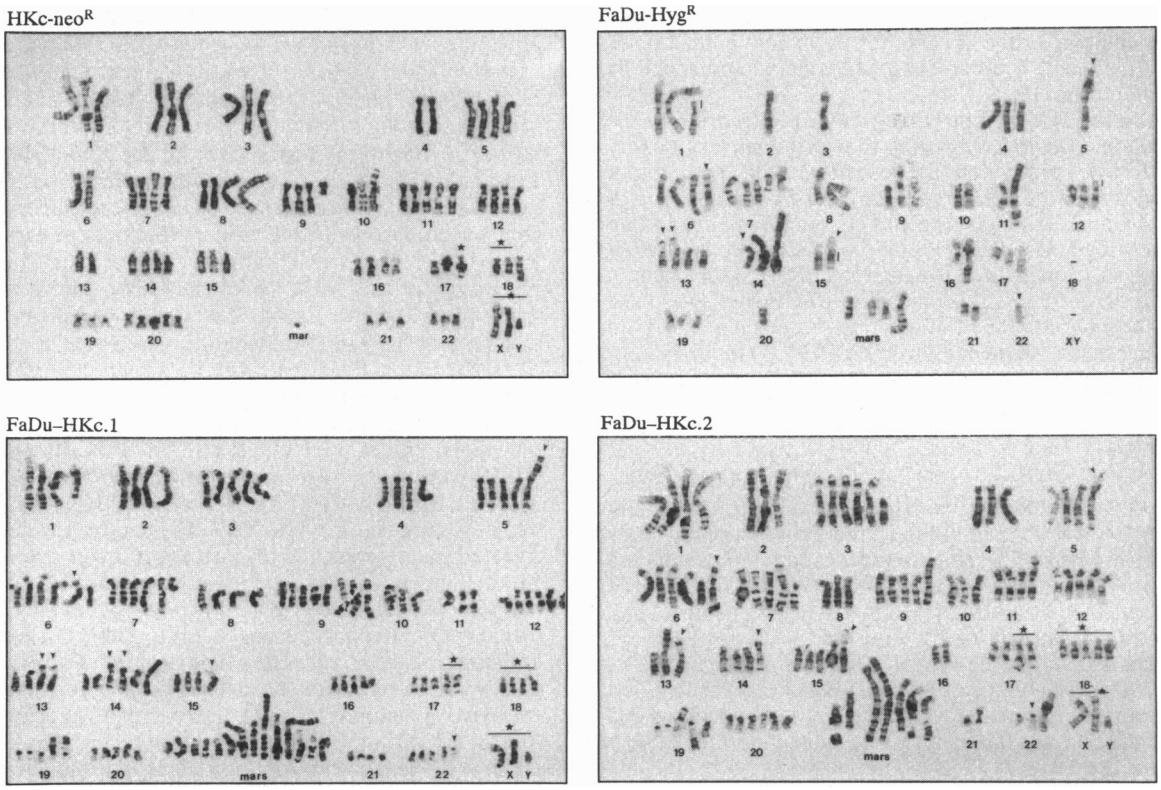

FIG. 2. G-banded karyotypes of parental and somatic cell hybrid cell lines. (*Upper Right*) For FaDu-Hyg^R, arrowheads indicate marker chromosomes identified in the hybrids. Vertical lines indicate regions containing sequences from chromosome 18, as identified by fluorescence *in situ* hybridization using a probe specific for the entire human chromosome 18. The metaphase shown contains the modal number of 55 chromosomes. (*Upper Left*) For HKc-neo^R; stars and horizontal lines indicate chromosomes and groups of chromosomes identified in the hybrids. This metaphase contains 78 chromosomes, not the modal number of 80 chromosomes (*Lower Left*). For FaDu–HKc.1, arrowheads indicate chromosomes from the parental FaDu-Hyg^R line; stars and horizontal lines indicate single chromosomes and groups of chromosomes derived from the HKc-neo^R parental line. This metaphase contains 121 chromosomes, not the modal number of 124 chromosomes (*Lower Right*). For FaDu–HKc.2, arrowheads indicate chromosomes from the parental FaDu-Hyg^R line; stars and horizontal lines indicate single chromosomes and groups of chromosomes derived from the HKc-neo^R parental line. This metaphase contains 103 chromosomes, not the modal number of 104 chromosomes.

whereas HKc-neo^R, FaDu–HKc.1, and FaDu–HKc.2 cells did not proliferate in the presence of 40 pM TGFβ1 (data not shown).

The simplest interpretation of this result is that responsiveness to TGFβ1 is under the direct control of a recessive gene or genes (24). The antimitogenic signal of TGFβ1 appears to be mediated primarily by two transmembrane receptors of $\approx$53 kDa (TβR-I) and 80 kDa (TβR-II). TβR-II is a member of the activin-receptor family of protein-serine/threonine kinases (25, 26). Use of a chemical crosslinking assay had shown (12) that FaDu cells expressed normal levels of TβR-I but a reduced level of TβR-II protein when compared with non-neoplastic human keratinocytes. Thus, it was possible that TβR-II was encoded by the recessive gene in question. We quantified the expression of TβR-II mRNA by using an RNase protection assay. The parental lines, FaDu-Hyg^R and HKc-neo^R, as well as the two hybrid lines FaDu–HKc.1 and FaDu–HKc.2 expressed approximately equal levels of TβR-II transcripts (Fig. 4). Thus, resistance of FaDu-Hyg^R to TGFβ1 cannot be ascribed to loss of the TβR-II gene, although suppression of its expression at the posttranscriptional level, or alterations of its function in another manner, cannot be formally excluded at this point.

Because the phenotype of the two hybrid lines indicated that TGFβ resistance was primarily a recessive trait, we undertook a systematic comparison of the karyotypes of the two hybrid lines FaDu–HKc.1 and FaDu–HKc.2 with that of FaDu-Hyg^R in order to identify homozygous deletion of chromosomal regions. A limitation of this approach is that chromosomal regions included in uncharacterized marker chromosomes might be underscored. G-banding revealed

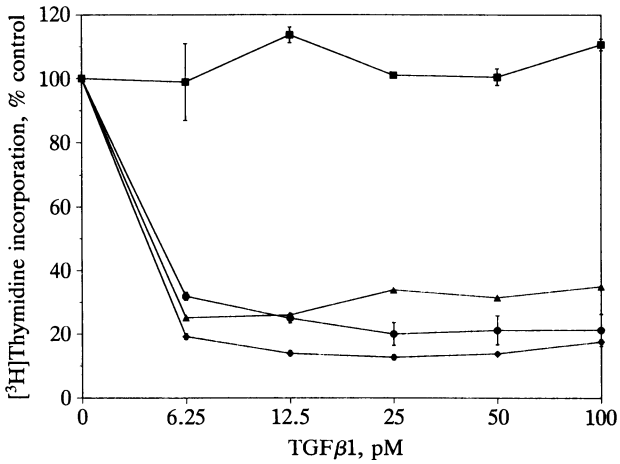

FIG. 3. Effect of TGFβ1 on DNA synthesis in parental and hybrid lines. Confluent cultures of HKc-neo^R cells and FaDu-Hyg^R cells, as well as the two FaDu–HKc hybrid lines, in 24-well cluster dishes were treated with TGFβ1 at the concentrations indicated for 20 hr. Cells were then incubated with [*methyl*-^{3}H]thymidine (specific activity, 0.5 Ci/mmol; 1 Ci = 37 GBq) at 5 μCi/ml for 2 hr at 37°C, and the amount of trichloroacetic acid-precipitable, hot perchloric acid-soluble radioactivity was determined as described (5). Means and SEM of three experiments are shown.

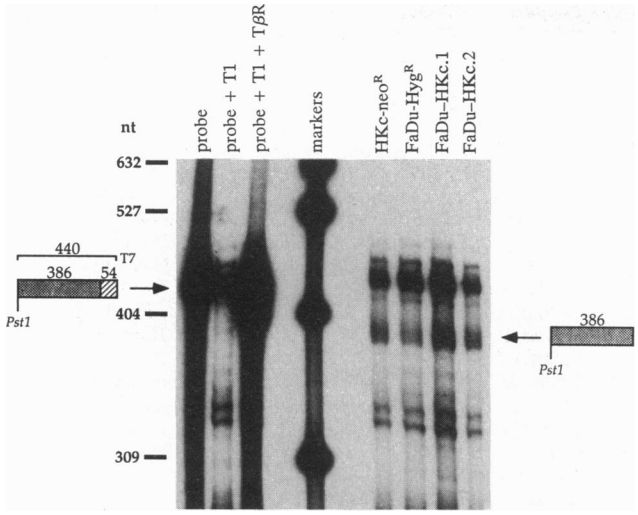

FIG. 4. Expression of TβR-II mRNA in parental cells and somatic cell hybrids as determined by RNase protection assay. ^{32}P-labeled *Msp* I fragments of pBR322 were included as size markers. A protected RNA fragment of 386 nt was present in RNA from HKc-neo^R FaDu-Hyg^R, FaDu–HKc.1, and FaDu–HKc.2 cells. Control lanes: probe, 440-nt antisense cRNA probe alone; probe + T1, 440-nt probe treated with RNase T1; probe + T1 + TβR, 440-nt probe hybridized with sense cRNA fragment, followed by treatment with RNase T1.

that only single copies of regions 2p, 3p, and 5p were present in FaDu-Hyg^R cells, whereas both hybrids carried four or five copies of each of these chromosomal regions. Furthermore, no copies at all of chromosomal region 18q were identified in FaDu-Hyg^R cells. However, by fluorescence *in situ* hybridization of metaphase spreads with a painting probe for the entire chromosome 18, a portion of 18q was identified in a marker chromosome previously thought to be derived solely from chromosome 15. Three additional copies of 18p were present in other derivative chromosomes. Review of the G-banding of the marker chromosome that contained 18q sequences suggested that the distal portion of this region was lacking. In summary, FaDu-Hyg^R cells display a homozygous loss of a region that extends at least from 18q22 to 18qter. Both hybrids carried multiple copies of intact chromosome 18, derived from the HKc-neo^R parent. Thus, 18q22–18qter may contain a gene or genes that mediate the cellular response to TGFβ1. This finding is particularly interesting for several reasons.

First, microcell transfer of chromosome 18 into SW480 colon carcinoma cells partially restored their responsiveness to TGFβ1 (27), whereas chromosomes 5 and 15 (27) and chromosome 11 (B. E. Weissman, T. M. Gilmer, and M.R., unpublished observations) did not have this effect.

Second, several recent studies have shown that chromosomal breaks, deletions, or interstitial losses of 18q occur with high frequency (40–100%) in SqCC lines as well as primary SqCCs of the head and neck, providing further support for the idea that the loss of one or more genes on the long arm of chromosome 18 play an important role in the pathogenesis of these neoplasms (28–30). Chromosome 18q contains a number of known genes that may well play a role in controlling keratinocyte growth and differentiation. These include *DCC* and *BCL2*. *DCC* encodes a neural-type of cell adhesion molecule that is frequently deleted in late colonic adenomas and invasive colorectal carcinomas and is thought to represent a tumor-suppressor gene (31). *BCL2* inhibits programmed cell death and promotes tumor growth (32, 33). Thus, *BCL2* may be located close to the breakpoint of chromosome 18 in FaDu cells and activated by translocation to the vicinity of a strong heterologous promoter on, for example, chromosome 15.

An alternative interpretation of the results of our fusion experiment is that TGFβ1 resistance is caused by a dominant oncogene that is present in FaDu-Hyg^R cells and suppressed in the hybrid lines (24). If this were the case, TGFβ1-resistance would have to be the result of the combined activation of a dominant oncogene and loss of a suppressor gene. Pratt *et al.* (34) showed recently that such situations can occur in living cells. These investigators transfected simian virus 40-immortalized human uroepithelial cells with the EJ mutant *ras* oncogene. Some of the clones that overexpressed the Ha-*ras* gene were tumorigenic whereas others were not. Fusion of a tumorigenic clone with a nontumorigenic clone gave rise to nontumorigenic hybrids, which continued to express the mutant *ras* gene. Tumorigenic revertants of these hybrids had lost chromosomal regions associated with known tumor-suppressor genes (34). In our case, a candidate oncogene is the p53 tumor-suppressor gene, which is expressed at high levels in FaDu cells due to a point mutation in codon 248 (Arg → Leu) (16). The p53 gene is inactivated in most SqCCs and has undergone an activating mutation in a subset of these tumors (16). Although expression of the wild-type p53 gene in a SqCC line that lacks endogenous p53 did not restore its responsiveness to TGFβ1 (L. Brenner and M.R., unpublished observation), transfection of a mutant form of p53 into TGFβ1-sensitive mouse keratinocytes or human bronchial epithelial cells caused partial resistance to TGFβ1 (35, 36). Thus, even though wild-type p53 may not be required for cells to respond to TGFβ1, mutant forms of the protein may interfere with TGFβ1 signaling in a dominant fashion (35, 36). As expected, FaDu-Hyg^R cells expressed elevated levels of Ab240-reactive mutant p53 protein (Fig. 5). In contrast, HKc-neo^R cells did not express high enough levels of p53 to be detectable by Western blot, presumably because wild-type p53 is rapidly degraded to undetectable levels because these cells express the E6 oncoprotein of HPV16 (37). However, mutant p53 protein was also undetectable in either of the two hybrid lines by Western blot (Fig. 5) and by ELISA (Oncogene Sciences, Mineola, NY). FaDu-Hyg^R cells expressed 1.11 ± 0.3 ng of mutant p53 per mg of protein (mean ± SEM of two experiments), whereas none was detectable in extracts of HKc-neo^R, FaDu–HKc.1, or FaDu–HKc.2 cells. To determine whether transcriptional or posttranscriptional mechanisms accounted for this reduced expression, we examined HKc-neo^R, FaDu-Hyg^R, FaDu–HKc.1, and FaDu–HKc.2 cells for the presence of wild-type and mutant p53 DNA and mRNA by sequencing PCR-amplified cDNA and cRNA fragments that encompassed the point mutation at codon 248 in exon 7. At the genomic level, both wild-type and mutant p53 DNA sequences were detected (data not shown). As illustrated by sequencing of cRNA (Fig. 6), FaDu-Hyg^R cells expressed only the mutant p53 allele. In contrast, FaDu–

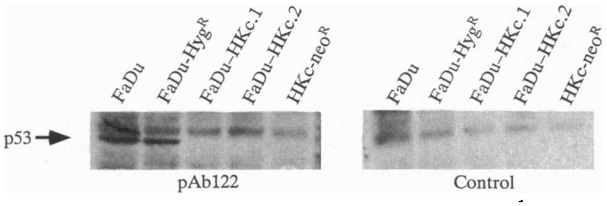

FIG. 5. Expression of mutant p53 protein in parental and hybrid cell lines as determined by Western blot analysis with the anti-p53 mouse monoclonal antibody Ab122 (Pharmingen, San Diego, CA), the mutant p53-specific antibody Ab240 (Oncogene Science) or an isotypic nonspecific mouse control IgG, as previously described (16, 36). FaDu-Hyg^R cells expressed Ab240-reactive mutant p53, as did the parental FaDu cells. In contrast, neither HKc-neo^R cells nor the two hybrid lines expressed detectable amounts of p53.

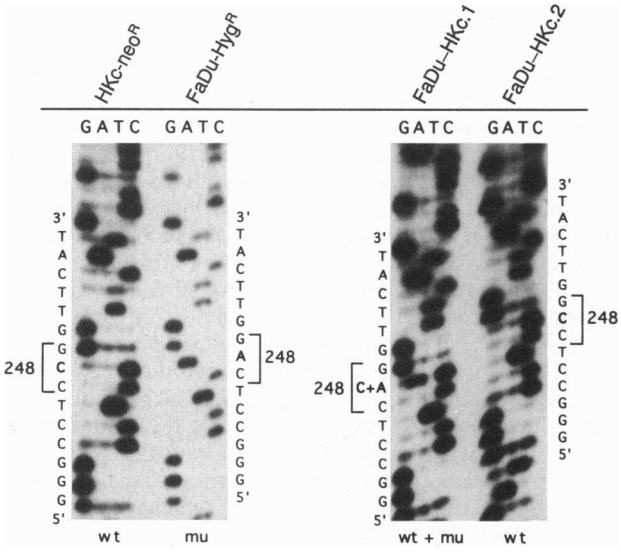

FIG. 6. Sequencing of PCR-amplified p53 transcripts in parental and hybrid cell lines. Exon 7 of the p53 gene, which includes the codon 248 that is mutated in FaDu-Hyg^R cells, was amplified by PCR and sequenced. wt, Wild type; mu, mutant.

HKc.1 cells expressed both wild-type and mutant p53 mRNA, whereas FaDu–HKc.2 cells expressed only wild-type mRNA (Fig. 6). Thus, in the case of FaDu–HKc.2 cells, the lack of expression of mutant p53 protein was the result of loss of the mutant allele from the FaDu-Hyg^R fusion partner. On the other hand, FaDu–HKc.1 cells did express mutant p53 mRNA, but the level of mutant p53 protein was strongly reduced (Fig. 5). Given that approximately equal amounts of wild-type and mutant cRNA were obtained by RNA PCR (see Fig. 6), suppression at a posttranscriptional level is probably the most likely explanation for the low level of expression of mutant protein. As many mutant forms of p53 have been shown to be degraded as a result of complex formation with the HPV16 E6 oncoprotein (37), the E6 gene contributed by the HKc-neo^R fusion partner and expressed in FaDu–HKc.1 cells may be responsible for the apparent absence of mutant p53 protein in these hybrid cells. However, as somatic cell hybrids of FaDu cells with HPV-negative mouse keratinocytes are also sensitive to TGFβ (38), it is also possible that degradation of p53 is driven by a normal cellular protein. In this sense, the phenotype of FaDu–HKc.1 cells resembles that encountered in somatic cells of patients with the Li–Fraumeni syndrome, in which both wild-type and mutant p53 proteins are expressed at very low levels, whereas, in the tumors that develop in these patients, the level of mutant protein is elevated (39). Thus, the recessive nature of resistance of FaDu SqCC cells to the antiproliferative effect of TGFβ1 may be the result of the loss of a dominant negative gene encoding a protein that normally plays a role in controlling p53 protein levels. Such a cellular protein might represent a novel tumor-suppressor gene.

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