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Localization of human *ERBA2* to the 3p22→3p24.1 region of chromosome 3 and variable deletion in small cell lung cancer

(oncogenes/thyroid hormone receptor)

H. DRABKIN*†‡, F.-T. KAO*§, J. HARTZ*, I. HART*, A. GAZDAR¶, C. WEINBERGER‖, R. EVANS**, AND M. GERBER*††

*Eleanor Roosevelt Institute for Cancer Research, 1899 Gaylord Street, Denver, CO 80206; †Division of Medical Oncology and University of Colorado Cancer Center, 4200 East 9th Avenue, Denver, CO 80262; §Department of Biochemistry, Biophysics and Genetics, and ††Division of Pulmonary Medicine, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262; ¶National Cancer Institute and Naval Hospital, Bethesda, MD 20814; ‖National Institute of Mental Health, Bethesda, MD 20892; and **The Salk Institute, P.O. Box 85800, San Diego, CA 92138-9216

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ABSTRACT **Human genes homologous to the v-*erbA* oncogene of avian erythroblastosis virus have been mapped to at least two human chromosomes. Recently, the *ERBA2* gene was shown to encode a thyroid hormone receptor and localized to chromosome 3 by using flow-sorted chromosomes. We now demonstrate that this gene is located at 3p22→3p24.1, using both somatic cell hybrids and *in situ* hybridization studies. Since this localization is close to the distal border of the small cell lung cancer (SCLC) 3p14→3p23 deletion, we undertook additional studies to examine the *ERBA2* gene in SCLC. Using somatic cell hybrids constructed from the SCLC line NCI-H182 as well as matched patient tumor and control tissue samples, we found that *ERBA2* is variably deleted. Therefore, *ERBA2* defines at the molecular level the distal border of the SCLC deletion and further implies that the putative suppressor gene is located centromeric of this locus. We also determined that, at least in NCI-H182, the 3p14 breakpoint is proximal to the constitutive 3p14.2 fragile site. These studies would indicate that the mechanism or initiation site of chromosomal rearrangement in SCLC is different from that which occurs during induction of the 3p14 fragile site by aphidicolin.**

Specific rearrangements of human chromosome 3 have been described in a variety of malignant and developmental disorders. Perhaps the best known rearrangement is the 3p14→3p23 deletion, which occurs in perhaps all cases of small cell lung cancer (SCLC) (1). Another example is in renal cell carcinoma, where both hereditary and spontaneous varieties contain alterations specifically in the 3p14→3p21 region (2–5). Other chromosome 3 rearrangements include (*i*) the Greig polysyndactyly craniofacial anomalies syndrome due to a 3;7 translocation, t(3;7)(p21.1;p13), and (*ii*) both inversions and translocations involving bands 3q21 and 3q26 in acute myelogenous leukemia with megakaryocyte hyperplasia (6, 7). Because of the colocalization of oncogenes and sites of chromosomal rearrangement (8), it was of interest to precisely localize the *ERBA2* gene on chromosome 3. This gene was isolated by its homology with the v-*erbA* gene from avian erythroblastosis virus and has been shown to encode a functional thyroid hormone receptor (9, 10). Since thyroid hormone is known to be important in cell growth and differentiation (11), alterations in *ERBA2* might be predicted, although no rearrangement of the human chromosome 3 or 17 thyroid hormone receptor genes has yet been demonstrated. Therefore, we sought to localize *ERBA2* and to determine if this gene might be rearranged in any of the chromosome 3 abnormalities mentioned above. We found that *ERBA2* localizes to 3p22→3p24.1, using a combination of somatic cell hybrids and *in situ* hybridization studies. This result suggested that *ERBA2* might be useful in the analysis of the SCLC 3p14→3p23 deletion. Using SCLC cell lines and direct patient material and isolating a derivative chromosome 3 [der(3)] previously reported to contain a 3p14→3p23 deletion, we conclude that *ERBA2* is variably deleted in SCLC samples. At the molecular level, this defines a region that must be telomeric to the critical segment containing a putative suppressor gene. The identification of an *Msp* I polymorphism should also facilitate genetic linkage studies to this region.

The isolation of the der(3) chromosome from cell line NCI-H182 also provided a means to examine the proximal extent of the deletion. We found that the breakpoint is proximal to the common 3p14.2 fragile site induced by aphidicolin. The fragile site appears to be closely related to the breakpoint in the hereditary renal cell carcinoma 3;8 translocation, t(3;8)(p14.2;q24.1). Therefore, it would appear that a different initiation site of chromosomal rearrangement is involved in the generation of at least some 3p deletions in SCLC.

METHODS

Cell Lines and Somatic Cell Hybrids. The SCLC cell lines NCI-H182, -H69, -H345, and -H417 were grown in Ham's F-12 medium containing 15% (vol/vol) fetal calf serum. The cell line GM2963 was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ) and was similarly grown in F-12 medium containing 15% fetal calf serum. Hybrids TL9542/UC-12-8 and 3;8/4-1 contain the der(3) and der(8) chromosomes from the hereditary renal carcinoma 3;8 translocation and have been described (12). Hybrid H3-4 contains the 3q− chromosome 3pter→3q21::3q26→3qter as the only identifiable human material isolated from acute myelogenous leukemia cells containing a t(3;3)(q21;q26) translocation (13). Hybrid 3;7/UC2E-1 contains the der(3) chromosome 7pter→7p13::3p21.1→3qter from the Greig polysyndactyly 3;7 translocation (ref. 6; H.D., unpublished data). Hybrid R10-4 contains the der(3) chromosome 17qter→17q25::3p21→3qter isolated from cell line GM2963. UCTP-2A3 contains an intact human chromosome 3 as the only identifiable human material and was provided by Charles Scoggins (Eleanor Roosevelt Institute for Cancer Research). Hybrids H3-4, 3;7/UC2E-1, and UCTP-2A3 were grown in F-12 medium containing 6% dialyzed fetal calf serum, which provides a nutritional selection for the der(3) chromosome. Hybrid R10-4 was grown in McCoy's 5A medium containing 10% fetal calf serum plus hypoxanthine (30 μM), aminopterin (0.4 μM), and thymidine (30 μM). Prior to expanding cells for

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Abbreviation: SCLC, small cell lung cancer.

‡To whom reprint requests should be addressed.

DNA, a subclone was picked and examined cytogenetically to confirm the presence of the derivative chromosome. A large number of anonymous DNA probes from human chromosome 3 have been tested with these hybrids to verify their chromosomal content (data to be presented elsewhere). No inconsistencies were detected.

To isolate the der(3) chromosome from the SCLC line NCI-H182, cells were fused with the Urd$^-$ CHO auxotroph, Urd$^-$C, as described (12). Hybrids that grew in the absence of uridine were screened for human aminoacylase I (Acy I) activity by using cellogel electrophoresis and the method of Voss *et al.* (14). Three separate hybrids (182/UC-7-3, 182/UC-10-4, and 182/UC-6-8) without human Acy I activity were found to cytogenetically contain the same der(3) chromosome by using G-11 staining and G-T-G banding as described (15, 16). Hybrids containing human Acy I activity were found to contain the apparently normal human chromosome 3, either with the der(3) chromosome (hybrid 182/UC-11-4) or without it (hybrid 182/UC-8-5).

Molecular Probes and DNA Analysis. The probe for *ERBA2* was pheA4, which was ^{32}P-labeled by the method of Feinberg and Vogelstein (17). The probe for the human c-*raf-1* (*RAF1*) 3′ flanking sequence was kindly provided by Tom Bonner (National Cancer Institute). The D3S3 probe was kindly provided by Ray White (University of Utah). Standard protocols were used for restriction enzyme digestion of human, hamster, and hybrid DNAs, agarose gel electrophoresis, and Southern transfer to Nytran (Schleicher & Schuell) nylon membranes (18). Filters were hybridized at 42°C in 45% (vol/vol) formamide containing 5× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate), 0.5% sodium dodecyl sulfate (SDS), 100 μg of denatured salmon sperm DNA per ml, and 2× Denhardt's solution [1× = 0.02% (wt/vol) Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin]. After hybridization, filters were washed with 0.1× SSC/0.1% SDS at 55°C (final conditions) and then used to expose Kodak XAR film for various times.

***In Situ* Hybridization.** The procedure for *in situ* hybridization routinely used in our laboratory has been described (19, 20). Briefly, human chromosomes were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes synchronized with bromodeoxyuridine and thymidine. For cell hybrids, actively growing hybrid cells were treated with colcemid (0.025 μg/ml) for 1 hr prior to harvest. Slides were made according to standard procedures. All slides were treated with RNase for 1 hr at 37°C, rinsed in 2× SSC, and dehydrated in ethanol. The chromosomes were denatured in 70% formamide/2× SSC at 70°C for 2 min and then dehydrated in ethanol. The probe was labeled to 1–2 × 10^8 cpm/μg with [^{3}H]dCTP, [^{3}H]dATP, and [^{3}H]dTTP by the random primer oligolabeling technique of Feinberg and Vogelstein (17). Hybridization was carried out at 37°C for 18 hr in 50% formamide/10% dextran sulfate/2× SSCP/1 mg of denatured salmon sperm DNA per ml (1× SSCP = 0.06 M NaCl/0.0075 M sodium citrate/0.01 M sodium phosphate). After hybridization, the slides were washed extensively in 50% formamide/2× SSC at 41°C and then in 2× SSC at 41°C, followed by dehydration in ethanol and drying in air. Slides were coated with Kodak NTB-2 nuclear track emulsion, stored at 4°C for 14 or 21 days, and developed in Kodak Dektol. The slides were stained with Hoechst H33258 (1 μg/ml in 2× SSC), rinsed, exposed to UV light, and then stained with 7% Giemsa (Fisher) in phosphate buffer (pH 6.8).

RESULTS

A Southern blot analysis using the somatic cell hybrid panel and the *ERBA2* probe is shown in Fig. 1. The human *Eco*RI fragments at 5.6, 2.2, 2.0, and 1.1 kilobases (kb) (lane a) can be distinguished from the rodent fragments in this experiment. The human gene is present in hybrid 3;8/4-1 (lane d), which contains 3p14.2→3pter material and, conversely, is absent in hybrid TL9542/UC2-12-8 (lane c), which contains only chromosome 3p14.2→3qter material. These two hybrids were derived from the hereditary renal carcinoma translocation, t(3;8)(p14.2;q24.1) (4, 5). Therefore, the *ERBA2* gene is located in the 3p14.2→3pter region, and no rearrangement is evident in this chromosomal disorder. Hybrid 3;7/UC2E-1 (lane g) contains the der(3) chromosome (7pter→7p13::3p21.1→3qter) from the Greig polysyndactyly syndrome 3;7 translocation (ref. 7; H.D., unpublished data) and hybrid R10-4 (lane f) contains the der(3) chromosome (17qter→17q25::3p21→3qter) from cell line GM2963. From studies using anonymous DNA probes that we and others have isolated, the chromosome 3 breakpoint in hybrid 3;7/UC2E-1 is telomeric to the breakpoint in hybrid R10-4 (unpublished observations). It can be seen that *ERBA2* DNA sequences are absent in both of these hybrids, further regionally localizing this gene to 3p21.1→3pter.

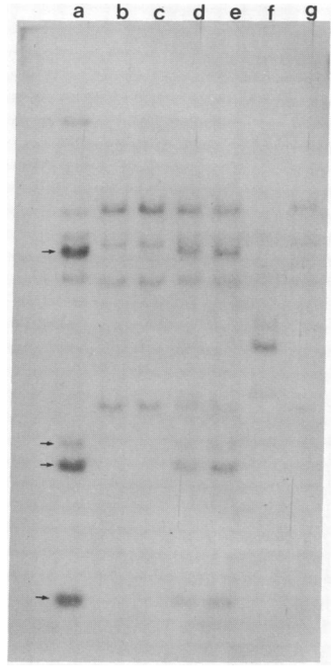

FIG. 1. Somatic cell hybrid regional localization of *ERBA2*. Approximately 5 μg of DNA was digested with *Eco*RI and hybridized with pheA4. The relevant chromosome 3 content of each hybrid is indicated in parentheses. Arrows indicate the human *Eco*RI fragments of 5.6, 2.2, 2.0, and 1.1 kb, which are readily distinguished from the rodent bands in this experiment. Lanes: a, human control; b, Chinese hamster ovary (CHO) control; c, TL9542/UC-12-8 (3p14.2→3qter); d, 3;8/4-1 (3pter→3p14.2); e, H3-4 (3pter→3q21::3q26→3qter); f, mouse–human hybrid R10-4 (3p21.1→3qter); g, 3;7/UC2E-1 (3p21.1→3qter). The 3p21.1 breakpoint in hybrid 3;7/UC2E-1 is telomeric to the breakpoint in hybrid R10-4.

To confirm and further define the *ERBA2* localization, we performed *in situ* hybridization studies on metaphase chromosomes from phytohemagglutinin-stimulated peripheral blood lymphocytes (Fig. 2). Twenty-eight metaphase cells with grains on chromosome 3 were analyzed in detail. The specific labeling of chromosome 3 among all cells examined was 15–20%. The background averaged 4.5 grains per spread. Of 127 total grains analyzed, 41 were located on chromosome 3, and of these, 66% (27/41) were at 3p22→3p24.1. This result confirms our findings from the somatic cell hybrid panel and places *ERBA2* telomeric to the chromosome 3p14→3p21 region implicated in renal carcinoma as well as significantly away from the Greig polysyndactyly 3p21.1 breakpoint.

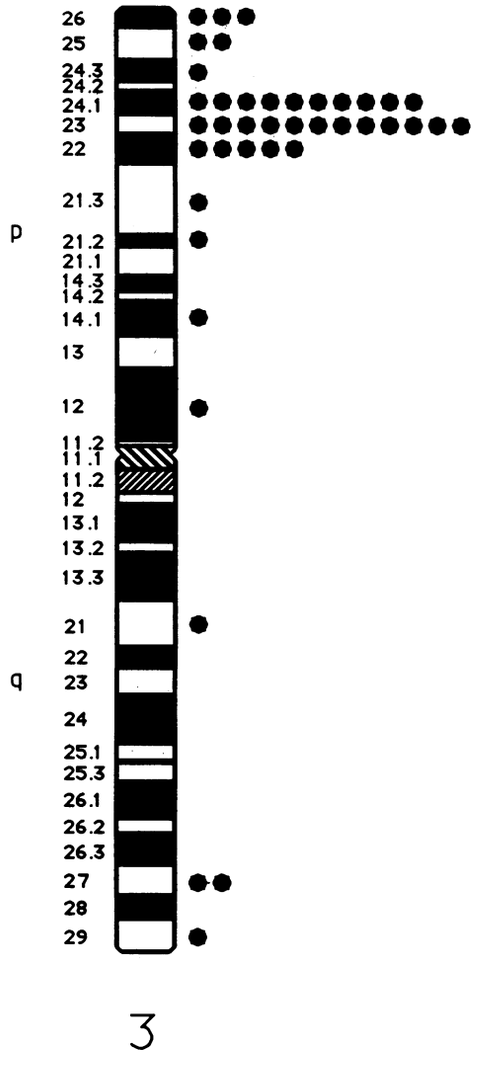

FIG. 2. *In situ* hybridization results showing localization of *ERBA2* to 3p22→3p24.1. Of 127 total grains, 41 were localized to chromosome 3; 66% of the chromosome 3 grains were at 3p22→3p24.1.

However, the *in situ* results place *ERBA2* cytogenetically near the distal (telomeric) border of the SCLC 3p14→3p23 deletion, so we therefore undertook additional studies to look for any alteration of *ERBA2* in this disorder and to further examine the distal border of the deletion.

A Southern blot analysis of three SCLC cells lines (NCI-H417, -H345, and -H69) digested with *Eco*RI and *Bam*HI demonstrated only germ-line fragments when hybridized with the *ERBA2* probe (data not shown). In an attempt to determine if *ERBA2* was deleted in SCLC, we hybridized DNA from the three SCLC cell lines simultaneously with *ERBA2* and a control probe mapped to the long arm, λ HD33, which we have isolated and localized to region 3q21→3q26 (Fig. 3). By visual inspection, the *ERBA2* bands (Fig. 3 *A* and *B*) in the SCLC samples (lanes 1–3) appear to be relatively at least as intense as in the control DNA (lane 4), especially when the long arm probe (Fig. 3*C*) is considered. Although densitometry tracings agreed with our visual interpretation, the proximity of the *ERBA2* fragments and the background smearing seen with this probe made it difficult to obtain a satisfactory baseline. While these results would suggest that *ERBA2* was not deleted in these cell lines, the aneuploid chromosomal content of these tumors (1) made this type of analysis unreliable, at least in our hands.

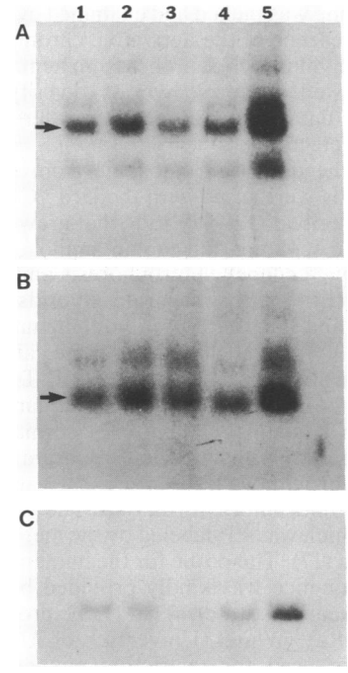

FIG. 3. Hybridization of *ERBA2* (*A* and *B*) and control long-arm probe, λ HD33 (*C*), to DNA from the SCLC cell lines NCI-H417 (lane 1), NCI-H345 (lane 2), and NCI-H69 (lane 3). Human control DNA is in lane 4 and was overloaded in lane 5. Arrows indicate the 5.6-kb and 2.0-kb *Eco*RI fragments in *A* and *B*, respectively.

The SCLC cell line NCI-H182 had been reported to contain only two copies of chromosome 3, one apparently normal and the other representing the deletion 3p14→3p23 chromosome (1)—i.e., 3pter→3p24::3p13→3qter. Therefore, we constructed somatic cell hybrids with this line, using the Urd^- CHO auxotroph, Urd^-C, to selectively retain human chromosome 3 on the basis of the uridine monophosphate synthetase gene located in the long arm (21). Three separate hybrids were found to contain the der(3) chromosome in the absence of the normal 3 chromosome. One hybrid contained both the der(3) and normal 3 chromosome, and another hybrid contained the normal 3 without the der(3) chromosome. Fig. 4 shows the karyotype of one of the hybrids, 182/UC2E-7-3, which contains the der(3) chromosome as the only identifiable human material in 100% of metaphase cells examined. The der(3) chromosome in the other hybrids

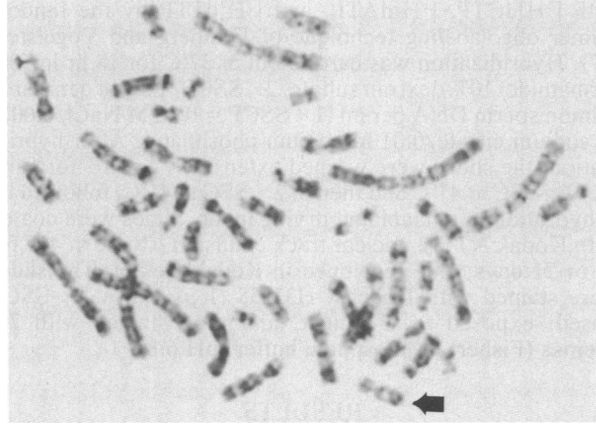

FIG. 4. Metaphase chromosomes from hybrid 182/UC-7-3 containing the der(3) chromosome isolated from cell line NCI-H182. The arrow indicates the der(3) chromosome, which was the only human material identifiable by cytogenetic analysis.

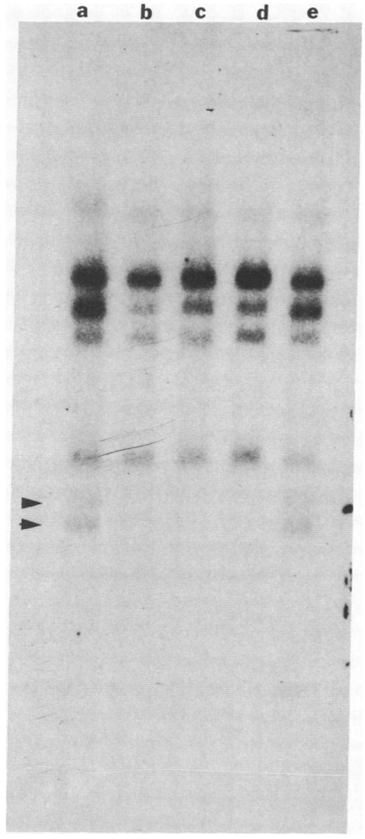

FIG. 5. *Eco*RI-digested DNA from somatic cell hybrids containing either a normal chromosome 3 or the der(3) chromosome isolated from NCI-H182 probed with *ERBA2*. Lanes: a, 182/UC-11-4 [der(3) and normal 3 chromosomes]; b, 182/UC-7-3 [der(3) chromosome]; c, 182/UC-10-4 [der(3) chromosome]; d, CHO; e, UCTP-2A3 (normal chromosome 3). The human 2.2- and 2.0-kb fragments are indicated by arrows. The 5.6-kb fragment was not well resolved from the CHO band on this gel. Identical results were obtained with hybrid 182/UC-6-8, which contains the der(3) chromosome in the absence of the normal chromosome 3 (data not shown).

appeared cytogenetically identical. We confirmed the presence of chromosome 3 DNA sequences in hybrid 182/UC2E-7-3, using additional anonymous probes that we have mapped to the long arm (data not shown). Fig. 5 shows that *ERBA2* is deleted from the der(3) chromosome from cell line NCI-H182. Of interest, we also found that the *RAF1* oncogene (3p24→3p25) was deleted as well as the DNA sequence *D3S3*, which we have previously localized to band 3p14.1 (22). This suggests that the 3p14→3p23 deletion reported for NCI-H182 may be more extensive or complex than described.

To examine direct tumor material with the *ERBA2* probe, we first identified an *Msp* I polymorphism of 17 and 20 kb with an approximate frequency of 36% and 64%, respectively, based on 18 unrelated individuals. We then examined the tumor and corresponding normal DNA from patients with SCLC (Fig. 6). Lanes 1–3 show the *Msp* I polymorphism in three normal individuals, and lanes 5 and 6 show polymorphism in two CHO–human hybrids containing independently isolated chromosome 3. Lane 4 is control CHO DNA. Three patients, G, T, and J, were heterozygous for the *Msp* I polymorphism. Both the loss and retention of *ERBA2* heterozygosity are clearly demonstrated in the tumor DNA from patients G and J, respectively. The results from the L and T tumors are less clear. The *Msp* I polymorphism is retained in the tumor DNA from patient T, although the signal intensity is reduced by approximately half. Similarly, although not heterozygous, the signal in the tumor DNA from patient L is reduced. In this case (patient L), equal amounts of DNA were loaded and confirmed by the use of a control probe (not shown). Thus, from data obtained using somatic cell hybrids, cell lines, and direct patient samples, we conclude that *ERBA2* is variably deleted in SCLC and that the proximal border of the 3p rearrangement in NCI-H182 is centromeric to the probe *D3S3* that has been localized to 3p14.1.

DISCUSSION

We have localized the *ERBA2* gene to region 3p22→3p24.1 using a combination of somatic cell hybrids and *in situ* hybridization studies. The only specific chromosome 3 rearrangement that we are aware of and might possibly involve *ERBA2* would be the SCLC 3p14→3p23 deletion. Whang-Peng *et al.* (1) found this region to be rearranged in all cases of SCLC examined. Molecular evidence for the loss of chromosome 3 heterozygosity has recently been demon-

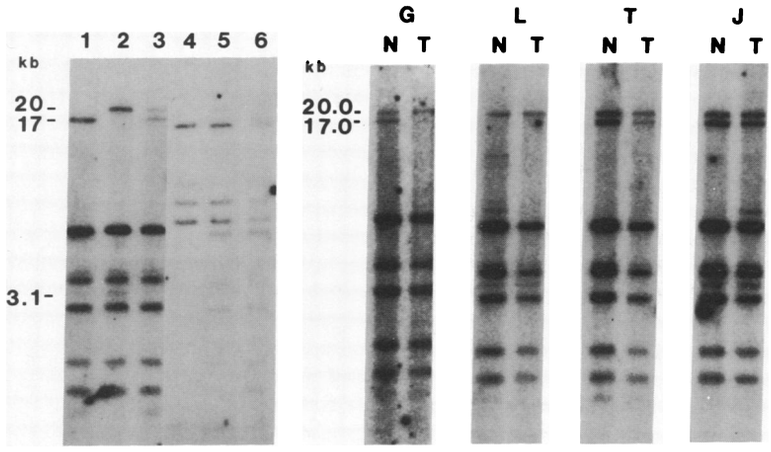

FIG. 6. *Msp* I digest of various DNA samples probed with *ERBA2*, showing 17- and 20-kb polymorphic fragments. (*Left*) Lanes 1–3 are digests of three different normal human genomic DNAs. Delineated are the 20-kb and 17-kb *Msp* I polymorphisms. The DNAs in lanes 1 and 2 show the homozygous alleles, and lane 3 shows DNA from a normal individual who is heterozygous at the *ERBA2* locus. The polymorphic frequency of the 20-kb allele is 0.64 and of the 17-kb allele is 0.36 by examination of 18 unrelated individuals. Lane 2 also shows a 3.1-kb band, which may represent another polymorphic fragment. Lane 4 shows DNA from CHO. Lane 5 shows hybrid DNA containing a normal human chromosome 3 as its only nonhamster DNA. The 20-kb allele segregates with this single chromosome 3 hybrid. Lane 6 shows hybrid DNA Y-195-4 containing a karyotypically normal chromosome 3 from SCLC cell line NCI-H69. The 17-kb allele segregates with this hybrid. (*Right*) Matched normal (N) and tumor (T) DNA from four patients (G, L, T, and J) with SCLC, digested with *Msp* I and hybridized with *ERBA2*.

strated in SCLC by using a limited number of probes from region 3p14→3p21 (23, 24). Similar findings have also been reported in non-SCLC as well (25), although we are unaware of a comparative chromosomal study between these two major types of lung cancer. The majority of the SCLC tumors are aneuploid for chromosome 3, and the rearrangements are frequently more extensive, although the 3p14→3p23 deletion is the smallest consistent abnormality. It is not clear what the critical region is within this large deletion. The proximal border contains the 3p14.2 fragile site (26) and is consistently altered in both hereditary and spontaneous renal carcinoma (2–5). Ibson *et al.* (27) described a SCLC that did not contain a deletion but rather contained a translocation involving 3p24, suggesting that this might be the most important region. Our observations that *ERBA2* is variably deleted suggest that the critical region containing a putative suppressor gene should be proximal to this locus. Mechanisms other than the loss of a suppressor gene, such as somatic mutation facilitated by an altered chromatin structure are of course possible. There is currently no experimental evidence that demonstrates clearly the presence of a functional suppressor gene on chromosome 3.

At the proximal border of the deletion, it is of interest that the breakpoint in the der(3) chromosome is proximal to the anonymous probe *D3S3*. This is consistent with our previous results with the hybrid Y-195-1, which contains the der(3) chromosome from the SCLC line NCI-H69 (22). This chromosome contains a 3p14→3pter deletion and was part of the mapping panel used to localize *D3S3* to 3p14.1 (22). Chromosome breaks at 3p14.2 induced by aphidicolin consistently occur telomeric to the *D3S3* probe (Tom Glover, personal communication). Furthermore, the hereditary renal carcinoma 3;8 translocation, which breaks cytogenetically at 3p14.2, is also telomeric to the *D3S3* locus (22). Therefore, in at least two instances in which the der(3) chromosome from different SCLC cell lines has been isolated away from the normal allele, the proximal breakpoint does not coincide with either the aphidicolin-induced fragile site or the renal carcinoma 3;8 translocation breakpoint. This raises the possibility that the fragile site may vary in different tissues. Laird *et al.* (28) have recently reviewed fragile site data in various species and present a strong hypothesis that the fragile site represents a region of late replication. If this is so, the actual region may vary considerably in different tissues or during development. Alternatively, the SCLC 3p alterations may not involve the fragile site *per se*. Answers to these questions will await the development of more probes and molecular cloning of the relevant breakpoints and fragile site DNA sequences.

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