Original Paper

Eur J Hum Genet 1993;1:156–163

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A PCR-Aided Transcript Titration Assay Revealing Very Low Expression of a Gene at Band 3p21 in 33 Cell Lines Derived from all Types of Lung Cancer

Abstract

We have developed a general PCR-based method to quantify the amount of a specific mRNA present in a given cell line or tissue. We applied this quantitative PCR to analyse the expression of D8, a human gene which we recently identified in the chromosomal region 3p21, the common deletion region of lung cancer. Our PCR-aided assay shows that in most lung-cancer-derived cell lines the amount of D8 transcripts is only 2% or less of that in normal lung tissue. The virtual absence of expression may imply some role of the gene in the development of lung cancer.

Key Words

Quantitative PCR
Chromosome 3
Lung cancer
Gene expression

Introduction

Heterozygous deletions of the short arm of chromosome 3 have consistently been found in various types of lung cancer, the shortest region of overlap of the deletions being band 3p21 [1–3]. Therefore, this band may be the site of a tumour suppressor gene. Recently, we identified in that region a gene, provisionally called D8 [4]. Whereas the D8 gene is ubiquitously expressed in human tissues, including lung, Northern analyses of 23 small-cell lung cancer (SCLC) cell lines failed to detect D8-mRNA. Conversion of mRNA to cDNA and subsequent amplification of the cDNA by the PCR showed, however, that D8-mRNA was detectable in all of these cell lines.

To assess the significance of this expression in SCLC, we have developed, along lines suggested earlier [5], a general PCR-aided titration method to quantify (low) levels of mRNA. Several such PCR-aided titration assays have been reported by others [6, 7]. These assays notably vary in their choice of internal standard. Our strategy involves the construction of a recombinant mRNA, identical to the original mRNA except for a relatively small insertion, to be used as an internal

Received:
June 29, 1992
Accepted:
October 29, 1992

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1018–4813/93/
0012–0156$2 75/0

standard. It can be reverse transcribed and amplified with the same oligonucleotide primers as the cellular RNA, but can be distinguished from the cellular mRNA by its slightly longer PCR product.

Here we describe this PCR-aided transcript titration assay, which can be easily applied to other genes, and we present the results obtained for D8-mRNA levels in cell lines derived from all types of lung cancer.

Materials and Methods

Origin of Cell Lines

The GLC-coded lung cancer cell lines were established from biopsies of primary tumours by Dr. Loe de Leij, Department of Clinical Immunology, University Hospital, Groningen, The Netherlands. Culture conditions were as described [8]. The NCI-coded lung cancer cell lines were established at NCI-Navy Medical Oncology Branch, Naval Hospital, Bethesda by Drs. A. Gazdar, H. Oie, and E. Russell.

Primers

Primers were home-made by the phosphite triester method using a commercial oligonucleotide synthesizer (Gene Assembler plus, Pharmacia). They were selected from two exons separated by at least one intron. Sequences (cDNA) for D8-specific primers were 5-'TACCTCATCCGCTATATGCC and 5'GCCTACTGTGGGCTTACAATGC. The resulting PCR product from cDNA was 439 bp, and from genomic DNA 1.2 kb.

Isolation of Total Cellular RNA

Cells were harvested by centrifugation (5 min, 1,000 *g*) when cultures were in log phase. For cell counting, the pellet was suspended in 1/50 vol culture medium and 1/50 vol of a buffer consisting of 4 m*M* $NaHCO_3$, 140 m*M* NaCl, 5.4 m*M* KCl, 1.0 g/l dextrose and 0.5 m*M* EDTA, pH 7.0. Cell clusters were suspended by repeated pipetting. A 20-µl aliquot was diluted 1:10 (v/v) with a vital dye (Türks solution, Merck), and the cells were counted using a haemocytometer (Büchner). The remaining suspension was centrifuged (5 min, 1,000 *g*) and the cell pellet solubilized in 10 vol of 6 *M* guanidine·HCl, 0.1 *M* Na-acetate, and sheared by repeated suction through a 21-gauge needle. RNA was precipitated by centrifugation (15 min, 10,000 *g*) after mixing the solution with 0.5 vol ethanol. The pellet was dissolved in 7 *M* urea, 100 m*M* NaCl, 10 m*M* EDTA, 0.1% SDS to an approximate RNA concentration of 1 mg/ml, extracted twice with 1 vol phenol/chloroform (1:1 v/v), and once with chloroform. RNA was again precipitated by the addition of 0.1 vol 3 *M* Na-acetate and 2 vol of ethanol, and pelleted by centrifugation (15 min, 10,000 *g*). Finally, it was dissolved in distilled water to an approximate concentration of 1 mg/ml. For the isolation of RNA from tissues the same method was used after mincing the sample in the guanidine·HCl solution. RNA concentrations were determined spectrophotometrically. The RNA was precipitated as described above, and stored at −80 °C.

Construction of Recombinant cDNA

The construction of recombinant D8-cDNA clones is outlined in figure 1. All cloning procedures were according to standard methods [9]. pB1.9 contains about 1.9 kb cDNA corresponding to the 3'-end of the D8-mRNA (fig.1), including the unique BstEII site 260-bp upstream from the polyadenylation site, in BlueScript SK. Small HaeIII restriction fragments, generated by disgesting pUC19 to completion with HaeIII, were ligated into the BstEII site. Recombinant plasmids were screened for inserts less than 200 bp by agarose gel analysis of EcoRI digests, and by PCR analysis using the D8-specific primers. Sequence analysis of one recombinant, using primers flanking the BstEII site (fig. 1), revealed an insert of 107 bp. PCR analysis of the original 1.9-kb cDNA and the recombinant with the D8-specific primers indicated in figure 1, gives rise to products of 439 bp and 546 bp, respectively.

Synthesis of Recombinant RNA

All buffers and solutions were made RNAse-free by treatment with diethylpyrocarbonate (Sigma). Solutions containing Tris base were filtered through a 0.2-µm nitrocellulose filter. Recombinant cDNA (2.5 µg) was linearized with SacII, and transcribed in vitro with T7 RNA polymerase (Pharmacia), starting from the T7-promoter lying just upstream from the multiple cloning site in the vector.

Synthesis was carried out in 200 µl buffer containing 40 m*M* Tris·HCl pH 7.9, 6 m*M* $MgCl_2$, 10 m*M* DTT, 500 µ*M* of each ribonucleotide, 100 units RNAsin (Pharmacia), and 40 units T7 RNA polymerase, at 37 °C for 1 h. cDNA was specifically degraded by a 20-min incubation at room temperature with 30 units RNAse-free DNaseI (Boehringer). DNaseI was then inactivated by heating at 80 °C for 5 min. The mixture was layered upon 2 ml of an aqueous solution of

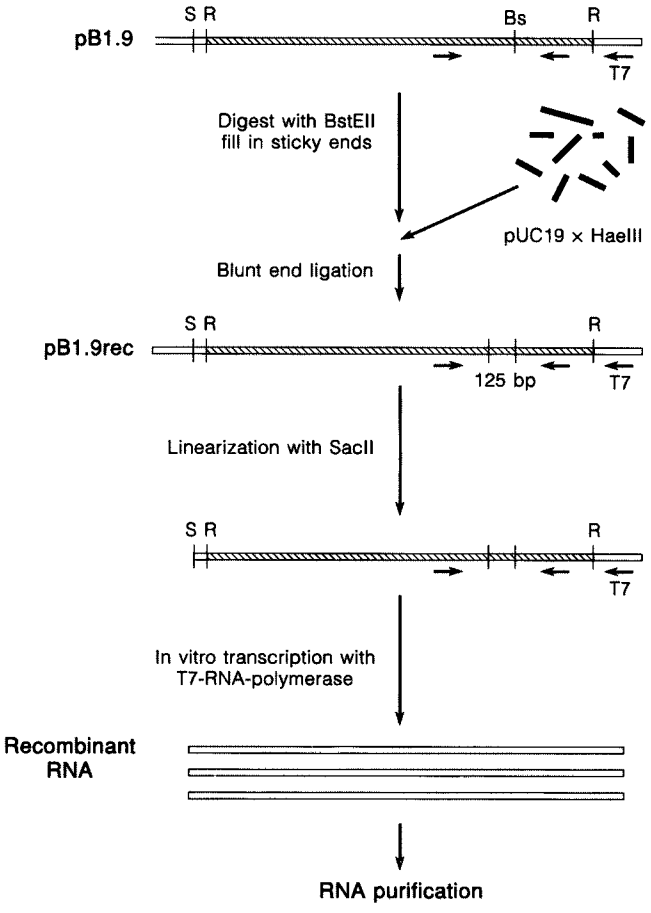

Fig. 1. Outline of the construction of the recombinant D8 cDNA clone, and the synthesis of the recombinant RNA. At the top the cDNA clone pB1.9 is shown, containing 1.9 kb of the 3′-part of the gene cloned into EcoRI site of BlueScript SK. A 102-bp HaeIII fragment of pUC19 is ligated into the BstEII site. Filling in of the 3′ protruding ends of the BstEII sites causes an additional 5-bp increase in length. The length of the recombinant RNA (pB1.9rec) is 1,935 nucleotides. R = EcoRI; Bs = BstEII; S = SacII; T7 = T7 RNA polymerase promoter present in the vector. Arrows indicate the approximate position and the direction of the D8-specific primers.

CsCl (0.96 g/ml) and EDTA (0.01 *M*), and centrifuged for 19 h at 35,000 *g* to remove all traces of cDNA. The RNA pellet was washed extensively with 70% ethanol at −20 °C, air dried, and dissolved in 200 µl distilled water. RNA concentrations were determined spectrophotometrically. About 10 µg of RNA was synthesized by this procedure. It was of a uniform length as determined by agarose gel electrophoresis (data not shown). From the nucleotide sequence of the recombinant plasmid, an exact length of 1,935 nucleotides could be derived for the RNA molecules. In order to check for contamination with cDNA molecules, 200 pg of the recombinant RNA product was used in an amplification reaction, with the D8-specific primers, while omitting reverse transcription. Due to some reverse transcriptase activity of the Taq polymerase a very small amount of PCR product of the expected length was produced; however, no PCR product at all was formed in the complete RT-PCR procedure when, prior to reverse transcription, the recombinant RNA sample was treated with RNase. Thus the RNA was not contaminated with recombinant cDNA.

Quantitative RT-PCR Procedure

Total cellular RNA (5 µg) was mixed with a variable amount of synthetic recombinant RNA (5 ng to 0.06 pg; indicated in the legends to the figures), 10 units RNAsin and 5 pmol of each primer, in a total volume of 10 µl. After heating the RNA at 95 °C for 5 min, the primers were annealed by incubating at 55 °C for 30 min. First-strand cDNA synthesis was carried out with 8 units MMuLV reverse transcriptase

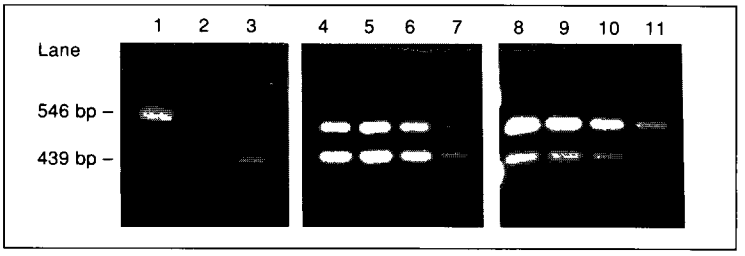

Fig. 2. PCR of mixtures of plasmid pB1.9 and pB1.9rec. Lane 1: amplification of 0.5 pg of pB1.9rec for 30 cycles; lane 2: amplification of 0.5 pg of each of the plasmids pB1.9 for 30 cycles; lane 3: amplification of 0.5 pg of pB1.9 for 30 cycles. Lanes 4–11: amplification of different mixtures of pB1.9 and pB1.9rec for various numbers of cycles. pB1.9 and pB1.9rec were mixed in equimolar amounts (lanes 4–7) and in a ratio of 3:1 (lanes 8–11). Each PCR mixture contained a total of 1 pg of cDNA. Amplification was carried out for 21 cycles (lanes 7 and 11), for 24 cycles (lanes 6 and 10), 27 cycles (lanes 5 and 9) or 30 cycles (lanes 4 and 8). The length of the pB1.9 and the pB1.9rec PCR products are 439 bp and 546 bp, respectively.

(Pharmacia) in the presence of 20 units RNAsin. Reaction conditions were as described by the manufacturers; cDNA synthesis was carried out in a total volume of 20 µl at 37 °C for 1 h. Without further purification, 4 µl of this mixture was subjected to 33 cycles of amplification in a buffer containing 10 m*M* Tris·HCl pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 200 µ*M* each of dATP. dCTP, dGTP and dTTP, 20 pmol of each primer, and 1.25 units of Taq polymerase (Boehringer), in a total volume of 30 µl. Each cycle consisted of 30 s denaturation at 92 °C, 1 min annealing at 58 °C and 1.5 min primer extension at 72 °C (the first denaturation step was for 2 min; the final extension step for 5 min). The amplified fragments were separated by electrophoresis on 1% NA agarose (Pharmacia)/2% NuSieve agarose (Research Organics) gels. Gels were photographed on Kodak Safety Film 5060. The relative intensity of the bands was either estimated visually from the titration series, or determined by densitometric scanning of negatives made from the same gel with different exposure times (UltraScanXL laser densitometer, Pharmacia).

To test the RT-PCR procedure, we routinely included primers specific for the APEH gene in the first-strand cDNA synthesis step [10]. The APEH gene is well expressed in a variety of control tissues, and in most, if not all, SCLC-derived cell lines [4, 11]. Only those RNA samples with a strong APEH-specific signal after amplification were subjected to the quantitative analysis of D8-mRNA.

Results

As can be seen from figure 2, a PCR analysis of a mixture of equimolar amounts of the 1.9 kb cDNA and the recombinant cDNA shows that under standard PCR conditions both templates are amplified with the same efficiency. This was not the case when we substituted a recombinant cDNA clone with a substantially longer insert (267 bp instead of 107 bp, leading to a product of 706 bp) for the recombinant clone described, although we varied extensively elongation time, temperature, and the buffer composition (data not shown). Since the two templates compete for the same primers, the ratio between the two PCR products is not affected by the number of cycles (fig. 2).

In our quantitative RT-PCR protocol, recombinant RNA is added to total cellular RNA isolated from a tissue sample or a cell line. After cDNA synthesis, a PCR with D8-specific primers is carried out for 33 cycles on volumes corresponding to 1 µg of total cellular RNA. A representative experiment is shown in figure 3. The cellular RNA, in this case iso-

Table 1. D8-mRNA levels in human tissues

Tissue type	D8-mRNA copies per 10 pg cellular RNA[1]
Lung (2)	250
Skin fibroblasts (3)	100
Tonsils	100
Ovary	60
Colon	60
Kidney	35
Muscle	12
Blood lymphocytes (3)	10
Liver	3

In those cases where the same tissue has been analysed several times (see numbers between brackets), the average value is given.

[1] See text for copy number calculations.

Table 2. D8-mRNA levels in small cell lung carcinoma-derived cell lines

SCLC cell lines	D8-mRNA copies per 10 pg cellular RNA[1]
GLC-1 (2)	4
GLC-2 (2)	3
GLC-3 (2)	8
GLC-4 (2)	4
GLC-7 (2)	1
GLC-8 (3)	11
GLC-14	5
GLC-16	2
GLC-20 (2)	8
GLC-28 (2)	4
GLC-34	3
GLC-42	2
GLC-44	3
NCI-H82	0.5
NCI-H187 (2)	1
NCI-H345	1

In case of multiple analyses of the same cell line (see numbers between brackets), the average value is indicated.

[1] See text for copy number calculations.

lated from human skin fibroblasts, gave rise to a PCR product of 439 bp. The recombinant RNA present in the samples gave rise to a PCR product of 546 bp. In lane 5 the PCR product from the cellular RNA is greatly in excess of the product from the added recombinant RNA. The converse is seen in lanes 1–3. In lane 4, the intensities of the PCR products are fairly close to each other. Taking into account the difference in length between the PCR products from the cellular RNA and from the recombinant RNA, respectively, their molar ratio was calculated from densitometry results to be 1.4 in this lane. This means that there is an amount of cellular RNA equivalent to 11 pg of the 1,935 nt long recombinant RNA. Since the full length D8-mRNA is about 3.5 kb [4], the amount of D8-mRNA per microgram of total cellular RNA must be approximately 20 pg. From the average molar mass of the ribonucleotides it follows that 1 pg of D8-mRNA contains 0.5×10^6 molecules. Thus, in the analysis shown in figure 3, about 10^7 D8-mRNA molecules are present per microgram of total cellular RNA.

D8-mRNA levels were determined for a variety of human tissues (table 1). Blood lymphocytes isolated from three different subjects contained between 1.3×10^6 and 0.7×10^6 D8-mRNA copies per microgram of total cellular RNA. For the fibroblast cell culture, the calculated number of D8-mRNA copies per microgram of total cellular RNA varied between 8.5×10^6 and 11×10^6 for three independent subcultures. The highest D8-mRNA level was measured in lung.

We analysed total cellular RNA isolated from 16 SCLC-derived cell lines. Figure 4 shows the experimental results for some of them as well as for normal lung tissue. Routinely, interpretation of the titration series was done visually. In those (9) cases where the ratio between the amount of the two PCR products was determined by densitometry as

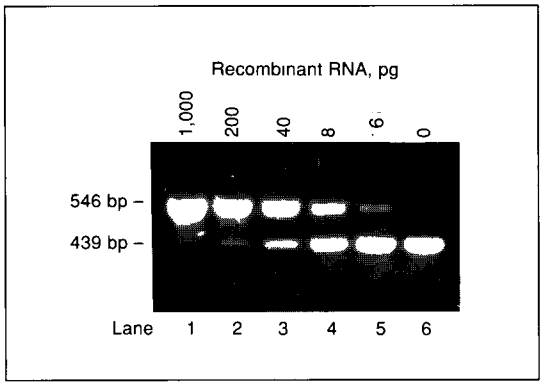

Fig. 3. Quantitative analysis of cellular RNA from cultured human skin fibroblasts. Above each lane, the amount of recombinant RNA added to 1 μg cellular RNA is indicated. The cellular RNA gives rise to the 439 bp PCR product, the recombinant RNA gives rise to the 546 bp PCR product.

well, the data were in good agreement. The results are summarized in table 2. It is obvious that in comparison with normal lung tissue the level of D8-mRNA in SCLC cell lines is extremely low.

For five of the SCLC cell lines (GLC-2, GLC-3, GLC-8, GLC-20, and NCI-H187) the amplification step was done in duplicate. In all cases the variation between the two results was less than 30%. For each of two SCLC cell lines (GLC-3 and GLC-8), two independent RNA isolations were carried out starting from independent subcultures. GLC-3 contained 1.0×10^6 and 0.6×10^6 copies of the D8 messenger per microgram of cellular RNA, respectively. For GLC-8 we measured 1.0×10^6 and 1.2×10^6 copies per microgram of cellular RNA, respectively.

We also included into our analysis 17 non-SCLC cell lines (table 3). With two exceptions, NCI-H157 and NCI-H1373, the range of D8-mRNA concentrations measured was similar to that found for the SCLC-derived cell lines (table 2).

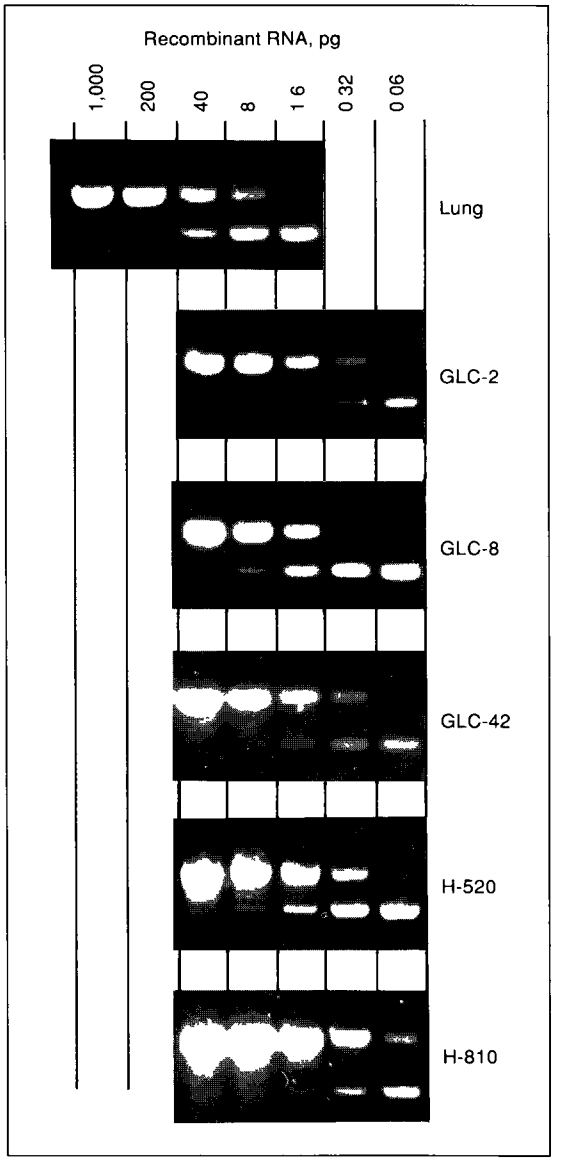

Fig. 4. Quantitation of D8-mRNA levels in cellular RNA isolated from normal lung tissue and from 5 cell lines derived from small cell lung carcinoma specimens. Above the lanes, the amount of recombinant RNA added to 1 μg of cellular RNA is indicated. Interpretation as for figure 3 (see text) results in values of 220, 3, 11, 2, 3, and 1 D8-mRNA copies per 10 pg of cellular RNA for lung, GLC-2, GLC-8, GLC-42, NCI-H520, and NCI-H810, respectively. GLC-2, GLC-8 and GLC-42 are SCLC cell lines; NCI-H520 and NCI-H810 are non-SCLC cell lines.

Table 3. D8-mRNA levels in cell lines derived from non small cell lung carcinomas

Non-SCLC cell lines	D8-mRNA copies per 10 pg cellular RNA[1]
Squamous	
GLC-P1	2
GLC-P2	4
NCI-H157	30
NCI-H226	3
NCI-H520	3
NCI-H1264	1
NCI-H1373	24
Adenosquamous	
NCI-H125	15
Adenocarcinoma	
GLC-A2	12
NCI-H23	0.5
NCI-H322	0.5
NCI-H358	6
Large cell	
NCI-H460	3
NCI-H661	0.5
NCI-H810	1
NCI-H1155	15
NCI-H1299	1

[1] See text for copy number calculations.

Discussion

In our approach, like in other PCR-aided transcript titration assays, the problem that the efficiency of the amplification of the transcript under study is not known and may not be constant in all cycles of the PCR is circumvented by the use of an internal standard which is amplified with the same set of primers as the DNA or mRNA under study [5–7, 12]. By using recombinant RNA instead of DNA as an internal standard, a possible variability in the efficiency of the reverse transcriptase reaction is addressed at the same time. Because the difference in length between the two templates competing for the same primers is relatively small, the ratio between the two products is independent of the number of cycles (fig. 2), as has also been clearly shown by others [7]. Even when an additional set of primers is included, e.g. for stimultaneous amplification of a second mRNA, the ratio between the two products is not affected (data not shown). We believe that our approach presented here to quantify D8-mRNA expression has the most general applicability, since it will always be possible to ligate a small extra sequence into a given cDNA. Preliminary results, using an identical approach, have been obtained for the gene coding for topoisomerase II [13].

Several RNA samples have been analysed in two or three independent experiments. These analyses demonstrated that our method is highly reproducible, as the results never differed by more than 30%.

Our procedure allows the detection of as little as 50,000 copies of recombinant RNA added to 1 μg of cellular RNA. Thus it is sensitive enough to detect about one target mRNA molecules per 20 pg of total cellular RNA.

The D8-gene is ubiquitously expressed (table 1) [4]. Unstimulated blood lymphocytes, liver, and muscle showed very low D8-mRNA levels. It should be noted that the APEH gene assayed simultaneously was well expressed. A very high amount of D8 mRNA was measured in three EBV-transformed lymphoblastoid cell lines (800–1,250 copies per microgram cellular RNA). This suggests that in normal tissues there might be some correlation between the presence of dividing cells and D8-mRNA levels.

The method presented in this paper gives an estimate of the amount of D8 mRNA present per microgram of total cellular RNA, and thus indirectly of the mRNA copy number per cell. Assuming an average content of 15–20 pg RNA per cell, the majority of the SCLC-derived cell lines contain 6 copies or

less per cell. The same holds true for the non-SCLC-derived cell lines. It is doubtful whether these very low levels of mRNA have any functional meaning. On the other hand, the copy numbers, also the somewhat higher ones found for NCI-H157 and NCI-H373, may relate to transcripts of a mutant allele.

The extremely low expression found in lung cancer cell lines does not represent the general situation in cancer cell lines. By Northern analyses, transcripts from D8 were detectable in a variety of tumors [4]. Therefore, the consistent finding of a very low gene expression or even a virtual absence of D8-mRNA transcripts in almost all lung cancer cell lines studied might suggest some role – be it as a cause or as a consequence – for the D8 gene in the development in bronchogenic carcinoma.

Acknowledgement

This work was supported by grant GUKC90-15 from the Dutch Cancer Society.

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Note Added in Proof

Sequence analysis of the D8-gene showed that at the amino acid level it has a 46.7% identity to the human UBA1-gene which encodes the ubiquitin activating enzyme (K. Kok, R. Hofstra, A. Pilz, A. van den Berg, P. Terpstra, C.H.C.M. Buys, B. Carritt, manuscript in preparation).