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THE c-*erb*Aβ THYROID HORMONE RECEPTOR

Expression and cDNA sequence analysis of the hormone-binding domain in human cancer cell lines

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The human c-*erb*Aβ protooncogene encodes a thyroid hormone receptor (comprising a hormone-binding domain and a DNA-binding domain) which modulates expression of specific genes, such as cell differentiation genes. Using the reverse transcription and polymerase chain reaction (RT-PCR) assay, significant expression of the c-*erb*Aβ gene was detected in the SiHa, CaSki, HeLa cervical carcinoma; Hep3B, PLC/PRF/5, Mahlavu hepatocellular carcinoma; HT-1080 fibrosarcoma cell lines; as well as in normal MRC-5 embryo lung and FS-4 foreskin fibroblast cell lines. However, the Molt-4 leukaemia and Raji Burkitt's lymphoma cell lines exhibited very low levels of c-*erb*Aβ expression. Single-strand conformation polymorphism analysis and direct sequencing of PCR products of the c-*erb*Aβ hormone-binding domain cDNAs of these cell lines revealed identical sequences, but differed from the published human placental c-*erb*Aβ sequence by five single base disparities. Sequencing of an aberrant fragment fortuitously amplified from the HT-1080 cDNA library demonstrated concordance with the cDNA of pregnancy-specific glycoprotein 4, which is related to the tumour marker, carcinoembryonic antigen.

The human c-*erb*Aβ protooncogene encodes a thyroid hormone receptor. Several related c-*erb*A complementary DNAs (cDNAs) have been characterized, including those from human placenta (1), testis (2), chicken embryo (3), rat brain (4), pituitary (5) and heart (6). Thyroid hormone receptors belong to a superfamily of cellular receptors for hormones (which include glucocorticoid, oestrogen and progesterone), as well as retinoic acid and vitamin D receptors (7). These ligand-bound receptors function as transcription factors by binding to specific hormone responsive element DNA sequences, thereby modulating the expression of specific target genes. The c-*erb*A gene product consists of a DNA-binding domain at the amino portion and a hormone-binding domain spanning the carboxyl portion.

The oncogenic counterpart, the v-*erb*A oncogene of avian erythroblastosis virus (AEV), differs from the avian c-*erb*A gene by short terminal truncations and 17 point mutations leading to 13 internal amino acid substitutions of which 9 occur in the hormone-binding domain (4, 8). While itself unable to transform susceptible erythroid precursor cells, v-*erb*A oncogene acts synergistically with v-*erb*B oncogene (encoding an abnormal epidermal growth factor receptor) present in AEV to aggravate erythroleukaemia induced by the latter. The v-*erb*A protein inhibits erythroid stem cell differentiation by occupying critical DNA binding sites, thereby precluding the activity of its wild-type counterpart (7–10). One mechanism by which the v-*erb*A oncogene blocks the differentiation programme of erythroblasts is to arrest transcription of the erythrocyte anion transporter gene (11). Furthermore, the v-*erb*A oncogene can abrogate the growth inhibition of embryo fibroblasts mediated by retinoic acid (12).

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The localization of the c-*erb*Aβ protooncogene to chromosome 3 p22-p24.1, a region frequently deleted in small cell lung carcinomas and other cancers, implies a putative tumour suppressor role of this gene (13). Considering this implication and that the majority of v-*erb*A mutations occur in the hormone-binding domain, our objective was thus to study the expression of the c-*erb*Aβ protooncogene and determine whether activating mutations of its hormone-binding domain could be uncovered in human cancers.

Material and Methods

Cell lines. Human cervical carcinoma cell lines analyzed were SiHa, CaSki and HeLa, which contain 1 copy, 500 copies of human papillomavirus (HPV) type 16, and 10–50 copies of HPV type 18 respectively (14). Hepatocellular carcinoma cell lines included Hep3B and PLC/PRF/5 lines with integrated hepatitis B virus DNA, and Mahlavu (15). The Raji Burkitt's lymphoma cell line (16), and the Molt-4 T-cell acute lymphoblastic leukaemia line (17), were also studied. MRC-5 human embryo lung and FS-4 foreskin fibroblast cell lines served as normal controls. All cell lines were cultured in minimum essential medium supplemented with 10% foetal calf serum. A cDNA library constructed from HT-1080 fibrosarcoma cells was purchased from Clontech (Palo Alto, CA, USA).

Extraction of cytoplasmic RNA. Cells were harvested, pelleted and resuspended in 10 mmol/l Tris-HCl (pH 7.5), 0.15 mol/l NaCl, 1.5 mmol/l $MgCl_2$ and 0.65% Nonidet P40. Following removal of nuclei by centrifugation, the cytoplasmic lysate was transferred to an equal volume of 7 mol/l urea, 1% SDS, 0.35 mol/l NaCl, 10 mmol/l EDTA, 10 mmol/l Tris-HCl (pH 7.5). The mixture was extracted with phenol and chloroform, and precipitated with ethanol (18).

Reverse transcription and polymerase chain reaction (RT-PCR). Based on the human placental c-*erb*Aβ cDNA sequence (1), three primer pairs ERA1/ERA2, ERA3/ERA6 and ERA5/ERA4 were synthesized (New England Biolabs, Beverly, MA, USA) and designed to amplify 438, 749 and 564 base pair (bp) fragments respectively, within the hormone-binding domain of the c-*erb*Aβ gene. PCR primers for the human p53 cDNA-specific amplification were purchased from Clontech (Table). RT-PCR was performed using a GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Norwalk, CT, USA). A 10 μl reaction volume comprising 1 μg cytoplasmic RNA, 1 × buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 5 mmol/l $MgCl_2$), 1 U/μl RNase inhibitor, 2.5 μmol/l random hexamers, 1 mmol/l each of the four deoxyribonucleoside triphosphates (dNTPs) and 2.5 U/μl Moloney murine leukaemia virus reverse transcriptase, was incubated at room temperature for 10 min, 42°C for 1 h, 99°C for 5 min and 4°C for 5 min. To the 10 μl synthesized cDNA was added 40 μl of a PCR mixture, providing final concentrations of PCR buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 2 mmol/l $MgCl_2$), 0.1 μmol/l of each primer pair and 2.5 U/100 μl Taq polymerase. This mixture was heated at an initial 95°C for 1 min, and then subjected to 30 cycles of 95°C denaturation for 30 s, 55°C (for c-*erb*Aβ primers) or 60°C (for p53 primers) annealing for 30 s and 72°C extension for 1 min. For the HT-1080 cDNA library, 0.1 μg of DNA template was used in a 50 μl PCR volume, and PCR with the ERA5/ERA4 primer pair was also performed at an annealing temperature of 45°C using a slightly modified protocol. Ten microlitres of PCR product were electrophoresed in a 2% agarose gel, stained with ethidium bromide. Strict laboratory procedures were adopted to prevent specimen contamination with 'carryover' DNA (14).

Extraction and amplification of PCR products from agarose gels. To obtain specific PCR products for single-strand conformation polymorphism (SSCP) and direct sequencing, amplified target fragments were excised from agarose gels, DNA eluted and reamplified. The excised bands were frozen in Ultrafree-MC tubes (Millipore, Milford, MA, USA), thawed and centrifuged. To the centrifu-

Table

DNA sequences of oligonucleotide primers for the Polymerase Chain Reaction and direct DNA sequencing

Target	Primer code (and orientation)	5′ → 3′ sequence (and location)	Target fragment (bp)
c-*erb*Aβ	ERA1 (+)	(1051) GTCAATGCCCCAGAAGGTG (1069)	438
	ERA2 (−)	(1488) CAGCAGGAAACTATCTTGGTA (1468)	
c-*erb*Aβ	ERA3 (+)	(941) GGGAGCTCATCAAAACTGTC (960)	749
	ERA6 (−)	(1689) GGAAGGAATCCAGTCAGTCT (1670)	
c-*erb*Aβ	ERA5 (+)	(899) AGAAGTCCATCGGGCACAA (917)	564
	ERA4 (−)	(1462) CTATTCTCTCAACACAGGCA (1443)	
p53	P53U (+)	(793) CTGAGGTTGGCTCTGACTGTACCACCATCC (822)	371
	p53D (−)	(1163) CTCATTCAGCTCTCGGAACATCTCGAAGCG (1134)	

(+): sense; (−): anti-sense.

gate was added TE buffer (10 mmol/l Tris-HCl, pH 8.0, 1 mmol/l EDTA), incubated at 37°C for 15 min and centrifuged. This volume was extracted with butanol and ether, precipitated with ethanol, washed and resuspended in TE buffer. Suitable dilutions of the eluted DNA were reamplified by PCR and electrophoresed.

Single-strand conformation polymorphism. To screen for DNA aberrations in the ERA1/ERA2 fragment, PCR-SSCP analysis was performed using a previously described protocol (19). Using T4 polynucleotide kinase and [γ-^{32}P]ATP, ERA1 and ERA2 primers were 5'-end-labelled and added at a final concentration of 0.1 μmol/l each to a 10 μl volume containing 1 × PCR-SSCP-buffer (20 mmol/l Tris-HCl, pH 9, 50 mmol/l KCl, 2 mmol/l $MgCl_2$, 0.1 mg/ml BSA), 0.125 mmol/l of each dNTP, 1 U Taq polymerase and 1 μl of agarose-eluted PCR-amplified cell line cDNA. PCR was subjected to 30 cycles each of 95°C for 20 s, 55°C for 30 s and 72°C for 1 min. PCR products were electrophoresed on non-denaturing, 5% polyacrylamide gels containing 10% glycerol, dried and exposed to autoradiography film.

Purification and direct sequencing of PCR products. The reamplified products were extracted with chloroform and ether, precipitated with polyethylene glycol, NaCl, and washed with 80% ethanol (19). Each PCR product template was sequenced at least twice in both directions with ^{32}P 5'-end-labelled PCR primers (including ERA4 primer as an internal sequencing primer for PCR products of the ERA1/ERA2 primer pair) using a dsDNA cycle didcoxy sequencing system (GIBCO BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions with modifications. Taq polymerase was employed to catalyse 20 linear amplification cycles each at 95°C for 30 s, 55 C for 30 s and 72°C for 1 min. The sequencing reactions were electro-phoresed in 8% polyacrylamide gels which were dried and autoradiographed.

Computer programmes. Using the DNASIS (eighth version) and PROSIS (fourth version) software programmes (Hitachi, Brisbane, CA, USA), nucleotide and amino acid sequences were compared against the GenBank and EMBL databases.

Results

Expression of c-erbAβ gene in human cell lines. Using ERA1 and ERA2 primers, 438 bp fragments of desired size were obtained from RT-PCR of all cell lines except Molt-4 and Raji (Fig. 1A, lanes 1–9). To exclude the possibility that Molt-4 and Raji cDNAs were not amplifiable by PCR, the cDNAs of all cell lines were subjected to PCR using primers for the p53 tumour suppressor gene as a control. p53 PCR target fragments of 371 bp obtained for Molt-4 and Raji (Fig. 2, lanes 10 and 11) authenticated the integrity of the Molt-4 and Raji RNAs and cDNAs. The only cell line which failed to express p53 by the RT-PCR assay was the Hep3B hepatocellular carcinoma line (20) (Fig. 2, lane 3). With the ERA3 and ERA6 primer pair, PCR products of the correct size of 749 bp were amplified from cDNA templates of all the cell lines except Molt-4, Raji and HeLa, while the FS-4 line was not tested (Fig. 1B). To increase detection sensitivity, bands were excised from the agarose gel at 749 bp positions expected for Molt-4 and Raji, DNA eluted and nested PCR performed using the ERA1 and ERA2 primer pair, generating 438 bp target fragments for these two cell lines (Fig. 1A, lanes 10 and 11). Using ERA5 and ERA4 primers, 564 bp frag-

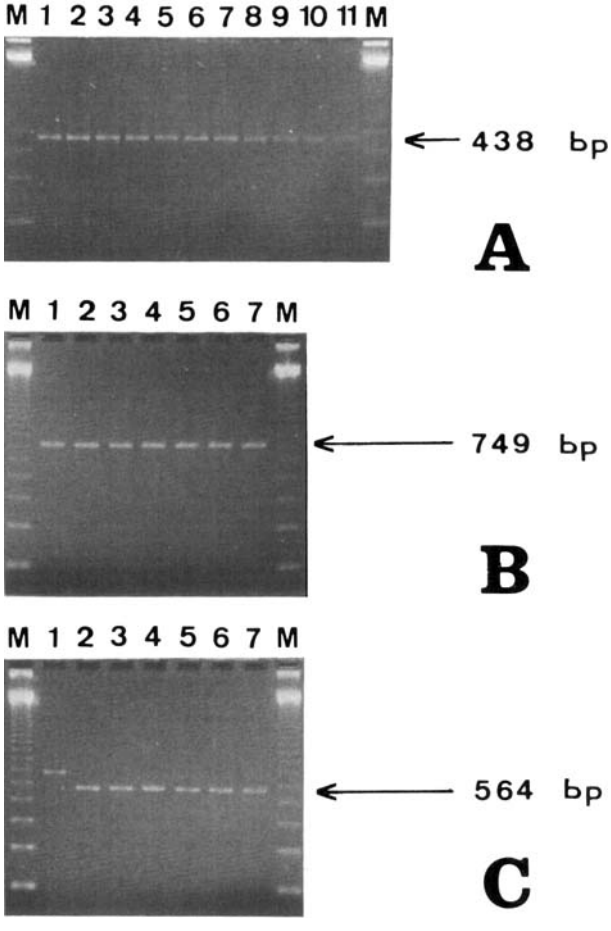


Fig. 1. Agarose gel electrophoresis of PCR products of gel-eluted amplified fragments using PCR primers for the c-*erb*Aβ protooncogene in human cell lines. (A–C) Lanes 1–7 correspond to HT-1080, Mahlavu, Hep3B, PLC/PRF/5, SiHa, CaSki and HeLa cell lines, respectively. M refers to 123 bp DNA ladder markers. (A) Lanes 8–11 represent MRC-5, FS-4, Molt-4 and Raji cell lines, respectively. 438 bp target fragments for all cell lines were amplified using ERA1 and ERA2 primers. However, the 438 bp bands for Molt-4 and Raji were obtained only after nested PCR using ERA1 and ERA2 primers on a template of ERA3/ERA6 PCR products, suggesting low c-*erb*Aβ gene expression. (B) 749 bp target fragments were generated by PCR of 7 cell lines using ERA3 and ERA6 primers. (C) 6 cell lines (lanes 2–7) yielded 564 bp fragments of expected size using ERA5 and ERA4 primers, except HT-1080 (lane 1) from which an aberrant-sized band of about 700 bp was amplified.

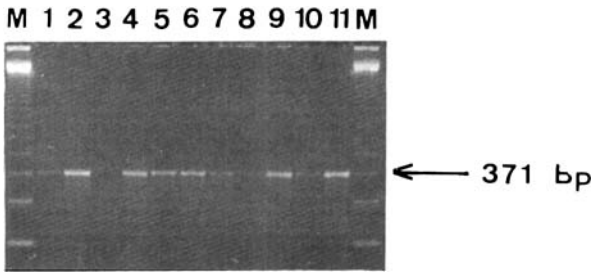


Fig. 2. Agarose gel electrophoresis of PCR products of the p53 tumour suppressor gene from cDNAs of human cell lines. Lanes 1–11 correspond to HT-1080, Mahlavu, Hep3B, PLC/PRF/5, SiHa, CaSki, HeLa, HeLa (heat-shocked), MRC-5, Molt-4 and Raji cell lines respectively, while lanes M are 123 bp DNA ladders. With the exception of Hep3B (lane 3), all the other cell lines, including Molt-4 and Raji (lanes 10 and 11), express the p53 gene.

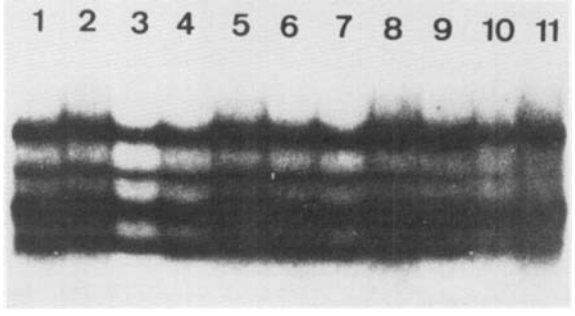


Fig. 3. Single strand conformation polymorphism analysis of ^{32}P-labelled PCR products of agarose gel-eluted 438 bp c-*erb*Aβ fragments using ERA1 and ERA2 primers. This autoradiograph of a non-denaturing polyacrylamide gel electrophoresis indicates absence of mobility shifts for HT-1080, Mahlavu, Hep3B, PLC/PRF/5, SiHa, CaSki, HeLa, MRC-5, FS-4, Molt-4 and Raji cell lines (lanes 1–11 respectively).

ments of expected size were amplified by PCR of cDNAs from all cell lines except HT-1080, Molt-4, Raji and HeLa, whilst FS-4 was not tested (Fig. 1C). PCR products of aberrant size of about 700 bp were amplified from HT-1080 instead of the expected 564 bp (Fig. 1C, lane 1).

Single-strand conformation polymorphism analysis. SSCP analysis of PCR products amplified using radiolabelled ERA1 and ERA2 primers revealed the absence of mobility shifts (Fig. 3) suggesting the lack of mutations within this fragment in the cell lines studied.

cDNA sequence analysis of c-erbAβ hormone binding domain. For Molt-4, Raji, HeLa and FS-4, only the corresponding 438 bp ERA1/ERA2 fragments were sequenced. For the rest of the cell lines, direct sequencing of the PCR products from all three c-*erb*Aβ primer pairs provided clearly readable ladders for the segment between the ERA5 and ERA6 primers from positions 899 to 1689. The sequence data obtained for all the cell lines were identical. In comparison to the published c-*erb*Aβ sequence of human placental cDNA (1), our data revealed five nucleotide differences. Three were found at nucleotides 1254 (G to A), 1353 (A to G) and 1651 (C to T) as illustrated in Fig. 4A–F. A T to C disparity was observed at nucleotide 1295, leading to isoleucine being substituted with threonine at amino acid residue 332 (Fig. 4A, B). A C to T disparity was detected at position 1636, resulting in a leucine to phenylalanine substitution at amino acid residue 446 (Fig. 4E, F).

Expression of pregnancy-specific glycoprotein gene in HT-1080 cells. Direct sequencing in both directions of the aberrant-sized 700 bp PCR product amplified from a cDNA library of the HT-1080 fibrosarcoma line revealed sequences incompatible with human c-*erb*Aβ cDNA. However, a computer search of the DNA database displayed virtually identical homology with pregnancy-specific glycoprotein 4 (PSG 4) cDNA. Clearly readable ladders were obtained for nucleotides 490–690 and 910–1 110 corresponding to the published cDNA sequence of PSG 4 (21), except for a C to G point mutational difference at nucleotide 1071 within the 3′ non-coding region (Fig. 5). The lack of the 564 bp c-*erb*Aβ target fragment may be explained by the likely absence of the full-length ERA5/ERA4 c-*erb*Aβ fragment in the inserts of the HT-1080 cDNA library. The high homology of the ERA5 and ERA4 primers to regions flanking a 700 bp segment within PSG 4 cDNA consequently permitted the PCR amplification of this PSG 4 fragment from the cDNA library, using a less stringent PCR annealing temperature of 45°C.

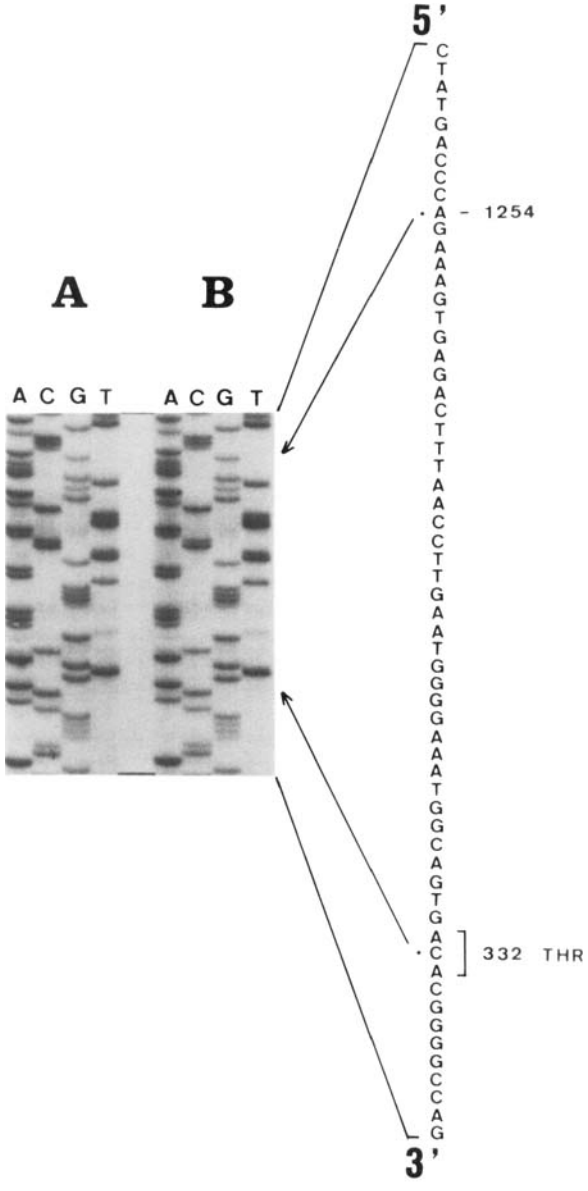
Discussion

In this study, the expression of the c-*erb*Aβ gene was detectable by the RT-PCR assay in 7 human cancer and 2 normal cell lines. In contrast, for Molt-4 and Raji lines, the failure to amplify c-*erb*Aβ target fragments using three separate c-*erb*Aβ-specific primer pairs confirms the extremely low level gene expression of c-*erb*Aβ in these two lines, expression being detectable only with the more sensitive nested PCR approach. The successful amplification of the 438 bp c-*erb*Aβ fragment from HeLa cDNA but not the larger 749 bp and 564 bp fragments also implies a certain degree of underexpression of the c-*erb*Aβ gene in HeLa cells. Molt-4 is a T-lymphoid leukaemia cell line with rearrangement of chromosome 7. Raji is a Burkitt's lymphoma cell line containing non-integrated Epstein-Barr virus, and has a chromosome 8 to 14 translocation which results in overexpression of the *myc* oncogene (16, 22). Interestingly, both Molt-4 and Raji are haematopoietic malignancies like the v-*erb*A-enhanced erythroleukaemia in which the subversion of c-*erb*A protein function abolishes cellular differentiation and promotes aggressive leukaemogenesis.

In a study using Northern blot analysis, Markowitz et al. (23) reported selective loss of c-*erb*Aβ expression in human colon carcinomas. This was attributed to suppression of gene transcription since no evidence of c-*erb*Aβ

gene deletion was found. In addition, Sakurai et al. (24) found significant expression of c-*erb*Aβ mRNA in human brain, prostate and thyroid, but expression was considerably much less abundant in tonsil, spleen, placenta, liver and kidney. Hypomethylation of the *erb*A1 gene in chronic lymphatic leukaemia (25), and increased c-*erb*Aβ mRNA in the 'sick euthyroid' syndrome (26) have also been reported. More work is needed to ascertain whether aberrations of c-*erb*Aβ gene expression play a significant role in the development of specific malignancies.

The gene transcript sequences of the c-*erb*ABβ hormone-binding domain were identical in all the cell lines studied but exhibited five nucleotide disparities compared with the published placental c-*erb*Aβ sequence. These sequence



ig. 4A–B.

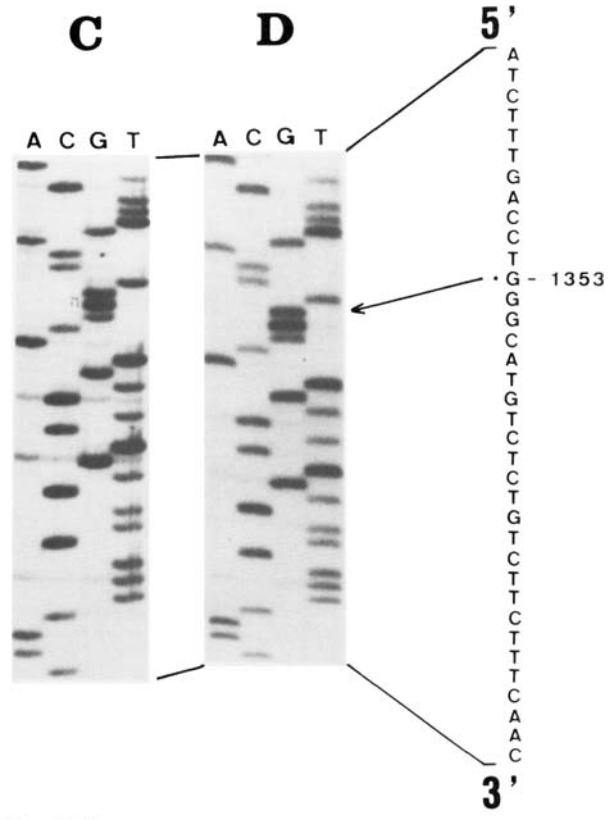


Fig. 4C–D.

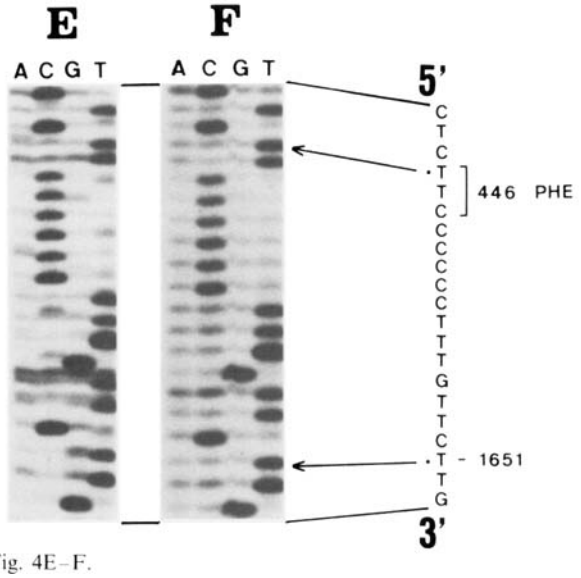


Fig. 4E–F.

Fig. 4. Direct DNA sequencing of c-*erb*Aβ PCR products reveals disparities with the published placental c-*erb*Aβ sequence (1). (A, B) Representative portions of the 438 bp ERA1/ERA2 fragments of MRC-5 and PLC/PRF/5 respectively, sequenced with the ERA4 primer, show a G to A difference at nucleotide 1254, and a T to C difference at codon 332 resulting in a threonine substitution. (C, D) An A to G difference at nucleotide 1353 is exemplified by the MRC-5 and HT-1080 cell lines, respectively (E, F). Parts of the 749 bp ERA3/ERA6 fragments of MRC-5 and PLC/PRF/5 lines respectively, sequenced with ERA6 illustrate a C to T difference at codon 446 coding for phenylalanine, and a C to T difference at nucleotide 1651.

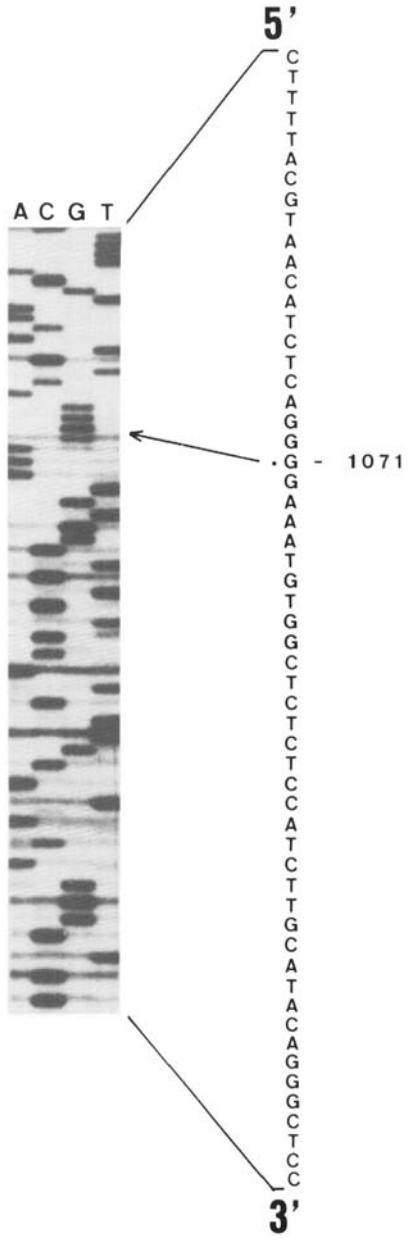


Fig. 5. Partial DNA sequencing of the aberrant 700 bp PCR fragment of the HT-1080 cell line using ERA5 and ERA4 primers. The sequence ladder shown was generated by sequencing with ERA4 primer, and was homologous to the 3′ non-coding region of the cDNA of pregnancy-specific glycoprotein 4 (21), except for a C to G disparity at nucleotide 1071.

differences have also been noted by Usala et al. (27). However, other gene mutations or alterations in the c-*erb*Aβ hormone-binding domain cannot completely be excluded, e.g. exon deletion variants due to intronic splice site mutations which have frequently been found in the p53 gene in various cancers (28). Although the DNA-binding domain of the c-*erb*Aβ gene was not investigated, this region could be a possible target for mutations.

While no cancer-associated c-*erb*Aβ mutations were detected in our series of cell lines, further work on a larger number of human cancers is required to firmly establish whether c-*erb*Aβ mutations are important in human tumourigenesis in the light of related research findings. For example, it was found that in a hepatocellular carcinoma, hepatitis B virus DNA integrates adjacent to a hepatocyte sequence homologous to v-*erb*A and other steroid receptor genes (29). Furthermore, the related retinoic acid receptor gene is rearranged and fused to the *myl* gene as a consequence of the t(15 ; 17) translocation event frequently encountered in acute promyelocytic leukaemia (30).

Finally, we documented the expression of the pregnancy-specific glycoprotein 4 gene in HT-1080 fibrosarcoma cells. First isolated and characterized from foetal liver, PSG 4 is a member of the family of pregnancy-specific glycoproteins which represent putative adhesion molecules related to the tumour marker, carcinoembryonic antigen (21). Using Northern blot analysis, Barnett et al. (31) have also demonstrated the expression of the homologous PSG 9 gene in HT-1080 cells.

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