

# Human Carcinoembryonic Antigen, an Intercellular Adhesion Molecule, Blocks Fusion and Differentiation of Rat Myoblasts

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**Abstract.** Human carcinoembryonic antigen (CEA), a widely used tumor marker, is a member of a family of cell surface glycoproteins that are overexpressed in many carcinomas. CEA has been shown to function *in vitro* as a homotypic intercellular adhesion molecule. This correlation of overproduction of an adhesion molecule with neoplastic transformation provoked a test of the effect of CEA on cell differentiation. Using stable CEA transfectants of the rat L6 myoblast cell line as a model system of differentiation, we show that fusion into myotubes and, in fact, the entire molecular program of differentiation, including creatine phosphokinase upregulation, myogenin upregulation, and  $\beta$ -actin downregulation are completely abrogated by the ectopic expression of CEA. The blocking of the

upregulation of myogenin, a transcriptional regulator responsible for the execution of the entire myogenic differentiation program, indicates that CEA expression intercepts the process at a very early stage. The adhesion function of CEA is essential for this effect since an adhesion-defective N domain deletion mutant of CEA was ineffective in blocking fusion and CEA transfectants treated with adhesion-blocking peptides fused normally. Furthermore, CEA transfectants maintain their high division potential, whereas control transfectants lose division potential with differentiation similarly to the parental cell line. Thus the expression of functional CEA on the surface of cells can block terminal differentiation and maintain proliferative potential.

**H**UMAN carcinoembryonic antigen (CEA)<sup>1</sup> (Gold and Freedman, 1965), is a member of a family of cell surface glycoproteins which appear at high levels in the blood of patients bearing a wide variety of tumors including colon, breast, and lung carcinomas (Zimmermann et al., 1988; Cournoyer et al., 1988), a phenomenon that has led to its widespread use as a tumor marker in the prognosis and management of colon cancer (Shuster et al., 1980; Terry et al., 1974). The basis for this marked elevation in blood levels of CEA is likely due to an increased accessibility to the vascular system in tumors, with some workers finding no increase in cellular CEA production (Abbasi et al., 1992) and others a true increase due to malignant transformation (Higashide et al., 1990); our evidence favors the latter (Boucher et al., 1989).

CEA is a member of the immunoglobulin supergene family (Williams, 1987; Paxton et al., 1987) consisting of a processed leader sequence, a V-like amino-terminal domain, six C2-set-like repeat domains in homologous pairs denoted

A1B1, A2B2, and A3B3, and a short COOH-terminal sequence which is processed to leave a glycoposphatidylinositol (GPI) bond to the external cell membrane (Fig. 2) (Beauchemin et al., 1987).

CEA has recently been demonstrated to act *in vitro*, at least, as a calcium-independent, homotypic, intercellular adhesion molecule (Benchimol et al., 1989). The adhesion function could be of relevance to the observed correlation between CEA family overproduction and cancer. We have suggested an instrumental model (Benchimol et al., 1989) based on the following rationale: the adult colonic epithelium consists of a monolayer of polarized cells which divide at the base of the crypts and move up the sides, undergoing a precise terminal differentiation as they progress to be eventually sloughed off into the lumen of the bowel; CEA production in these cells tends to be low and apical; early embryonic epithelium, on the other hand, is multilayered and CEA is found over the entire cell surface (Benchimol et al., 1989); colonic carcinomas also frequently consist of multilayered epithelial cells with CEA expressed over the entire cell surface.

We have suggested that this apparent reversion to an embryonic multilayered epithelial architecture in adult carcinomas could be due to an imbalance in the presentation of adhesion molecules on the surface of colonocytes arising from inappropriate production of CEA; this, in turn, could lead to a disruption of differentiation leaving cells with

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1. *Abbreviations used in this paper:* CEA, human carcinoembryonic antigen; CKS, CTP: CMP-3-deoxy-manno-octulosonate cytidyltransferase; CPK, creatine phosphokinase; GPI, glycoposphatidyl-inositol; HS, horse serum; pRB, retinoblastoma protein.

proliferative potential and providing a means for the selective outgrowth of cells with further oncogenic mutations (Benchimol et al., 1989). In this model, tissue architecture and cellular differentiation in heterogeneous cell populations is considered to be critically dependent on the accurate spatiotemporal display of a number of specific cell adhesion and factor receptor molecules on the cell surface (Edelman, 1984, 1986; Hatta et al., 1987; Fujimori et al., 1990). The effect of the ectopic introduction of CEA on the cell surface is envisioned as being potentially disruptive of normal cell-cell interactions which depend on these molecules.

As a test of this "instrumental" tissue architecture model, we have examined the effect of ectopic production of CEA in a well characterized differentiating system, the rat L6 myoblast. These cells, after reaching confluence in culture, undergo cellular alignment followed by fusion and the formation of multinucleated myotubes (Yaffe, 1968; Richler and Yaffe, 1970; for review see Wakelam, 1985). After proliferation to near confluence, the cells withdraw from the cell cycle before differentiation (Okazaki and Holtzer, 1966), a process which may be inhibited by growth factors (Linkhart et al., 1980; Lathrop et al., 1985; Florini et al., 1986), or oncogene activation (Olson et al., 1987; Payne et al., 1987; Gossett et al., 1988; Rahm et al., 1989). The process of cell cycle withdrawal is associated with an upregulation of helix-loop-helix transcriptional regulator proteins such as MyoD and myogenin (Wright et al., 1989), which have been shown recently to interact with the tumor suppressor retinoblastoma protein, pRB, a cell cycle regulator (Gu et al., 1993). Normal differentiation and fusion are associated with modifications of cell surface glycoproteins (Holland et al., 1984) and are inhibited by glycoprotein processing inhibitors (Gilfix and Sanwal, 1980; Holland and Herscovics, 1986; Spearman et al., 1987) and lectins (Den et al., 1975; Parfett et al., 1983). Other necessary modifications include alterations in the expression of intercellular adhesion molecules, such as a shift in the isoform of the neural cell adhesion molecule (Dickson et al., 1990), and changes in the level of cadherins (Knudsen et al., 1990; Pouliot et al., 1990). Thus, the L6 myoblast represents a well studied model system which depends on multiple signals, including factors, membrane bound receptors and adhesion molecules, for its differentiation program.

We have isolated stable CEA cDNA transfectant clones of L6 myoblasts and have measured their ability to fuse and differentiate. In agreement with the above model, independently selected transfectants producing relatively low amounts of CEA are unable to differentiate and fuse whereas nonproducers, control CEA anti-sense transfectants, myoblasts transfected with an adhesion-defective NH<sub>2</sub>-terminal deletion mutant of CEA (CEAΔN), and CEA transfectants treated with adhesion-blocking peptides, differentiate and fuse normally.

## Materials and Methods

### Cell Culture and Differentiation

The rat L6 myoblast cell line was the kind gift of Dr. P. C. Holland of the Montreal Neurological Institute. All experiments were performed using cells cultured for fewer than six passages after receipt. Cells were grown in monolayer culture in plastic tissue culture flasks or petri dishes contain-

ing D-MEM supplemented with 10% horse serum (HS) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The medium was changed every 2-3 d.

To promote fusion and differentiation, cultures were seeded at 10<sup>4</sup> cells/cm<sup>2</sup> in 100- or 150-mm tissue culture plastic petri dishes and grown in D-MEM + 10% HS without changing the medium for 5 d, after which the medium was replaced with D-MEM + 2% HS and the cells cultured for an additional 5 d. For fusion index determination, cells were fixed with 2.5% glutaraldehyde and stained with haematoxylin and eosin. The fusion index was calculated as the number of nuclei within cells containing three or more nuclei divided by the total number of nuclei counted. A minimum of 500 nuclei in four randomly selected areas was counted for each determination.

### Isolation of Transfectants

30 μg of full length CEA cDNA (Beauchemin et al., 1987) in the sense or antisense orientation in the expression vector, p91023B (courtesy of R. Kaufman, Genetics Institute, Boston, MA) or 30 μg of CEAΔN in pDCKdhfr (a gift from S. Oikawa, Suntory Limited, Osaka, Japan) and 3 μg of pSV2neo were cotransfected by electroporation with three pulses of 350 mV from 500 μF (Bio-Rad Gene Pulser) into 10<sup>8</sup> L6 myoblasts. CEAΔN has a deletion between the Pvu II and Acc I restriction sites in its cDNA, resulting in the removal of amino acids 32-106 in the 108-amino acid N domain of CEA, and the introduction of an arginine residue between tyrosine residues at positions 31 and 107 (or positions 65 and 141, including the leader [Beauchemin et al., 1987])—(Oikawa et al., 1991). After incubation for 10 min on ice, cells were grown for 48 h in regular growth medium after which positive clones were selected by the addition of 400 μg/ml of the cytotoxic drug Geneticin G-418 (Sigma Chem. Co., St. Louis, MO). To prevent the depletion of myoblasts with high fusion potential, cells were not permitted to achieve confluence or differentiate during transfection and selection. Clones were picked by micromanipulation under direct microscopic vision and were checked for their levels of CEA production using whole cell sonicates and a CEA double monoclonal antibody clinical kit (Abbott CEA-EIA Monoclonal; Abbott Laboratories, North Chicago, IL). This assay is highly specific and could not detect the truncated product of the CEAΔN cDNA. The latter was therefore analyzed using immunoblotting and immunofluorescence with anti-CEA polyclonal antibody. Verification of cell surface expression of CEA or CEAΔN was performed by immunofluorescence with mono- and polyclonal antibodies followed by both microscopic examination and analysis with a fluorocytometer (FACScan, Becton Dickinson, Immunocytometry Sys., Mountain View, CA) as described previously (Zhou et al., 1993).

### Immunoblot Analysis

Cells from monolayer cultures in the late exponential phase of growth were removed from culture dishes with PBS containing 15 mM sodium citrate, washed twice in cold PBS, and lysed by sonication in PBS at 4°C. The protein concentration of the sonicates was determined by the Bio-Rad protein assay (Bio-Rad Labs., Richmond, CA). 50 μg of each of the samples was boiled in Laemmli's buffer (Laemmli, 1970) for 4 min. Proteins were resolved by electrophoresis (Laemmli, 1970) on a 7.5% Tris-glycine-SDS-polyacrylamide gel under standard conditions. The proteins were transferred to a nitrocellulose membrane (Towbin et al., 1979) and the blot was probed successively with anti-CEA polyclonal rabbit antibody and alkaline phosphatase-conjugated Affipure goat anti-rabbit IgG(H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, PA). The labeled proteins were detected by reaction with 5-bromo-4-chloro-3-indolyl-phosphate-nitro-blue tetrazolium substrate (Promega Fisher Scientific, Montreal, Quebec).

### Cell Proliferative Potential

Replicate plates were seeded with 10<sup>4</sup> cells/cm<sup>2</sup> in 100-mm plates and cultured as above for the induction of differentiation. At intervals cells were removed with trypsin, rendered single cell suspensions and various dilutions plated in 60-mm tissue culture plates, using alpha-MEM (Stanners et al., 1971) plus 10% FCS as growth medium. After 10 d of incubation at 37°C, triplicate plates were fixed in 10% formaldehyde in PBS and stained with 0.1% methylene blue. Colonies of 25 cells or more were enumerated microscopically. The plating efficiency was calculated by dividing the number of colonies obtained by the number of nuclei plated (nuclei rather than cells to allow for cell fusion). The number of nuclei plated was determined from nuclear counts of replicates of the differentiating cultures which were

fixed and stained as described above for the fusion index determination. Values were normalized to the plating efficiency on day 2 for each cell line. The absolute plating efficiency was between 78–93% for all experiments.

### Creatine Phosphokinase Determination

Monolayer cultures of myoblasts at various stages of differentiation were harvested by scraping into ice cold PBS, sonicated, centrifuged at 15,000 rpm for 5 min and the supernatant kept at  $-70^{\circ}\text{C}$  before assay. 25–50  $\mu\text{l}$  of supernatant was analyzed for creatine phosphokinase (CPK) activity using the creatine kinase *N*-acetyl cysteine-activated ultraviolet system (Boehringer Mannheim Corp., Indianapolis, IN; GmbH Diagnostica) according to the manufacturer's protocol. The protein concentration of sonicates was determined by the protein assay (Bio-Rad Labs.). Results are expressed as  $\mu\text{mol}/\text{min}/\text{mg}$  protein.

### Northern Analysis

Total cell RNA was isolated by the guanidium isothiocyanate procedure of Chirgwin et al. (1979). 20  $\mu\text{g}$  of RNA was electrophoresed on 1.1 M formaldehyde, 1.5% agarose gels and transferred to nitrocellulose filters (Maniatis et al., 1982). Detection of myogenin and  $\beta$ -actin bands was accomplished with random primer  $^{32}\text{P}$ -labeled myogenin cDNA (courtesy of W. Wright, Houston, TX) or chicken  $\beta$ -actin cDNA hybridized at  $42^{\circ}\text{C}$  in  $5\times$  SSPE ( $1\times$  SSPE is 0.18 M NaCl plus 10 mM  $\text{NaPO}_4$  [pH 7.7] plus 1 mM EDTA),  $5\times$  Denhardt's solution, 50% formamide, 150  $\mu\text{g}$  of heat denatured salmon testis DNA, 0.5% SDS, for 18 h. The membrane was then washed in  $2\times$  SSPE + 1% SDS at  $20^{\circ}\text{C}$ , twice in  $1\times$  SSPE + 0.1% SDS at  $65^{\circ}\text{C}$ , and twice in  $0.1\times$  SSPE + 0.1% SDS at  $65^{\circ}\text{C}$ . To control for the amount of RNA added, blots were stripped and reprobed with the 18S rRNA specific oligomer 5'-ACGGTATCTGATCGTCTTCGAACC-3', end-labeled with  $^{32}\text{P}$  using T4-kinase (Maniatis et al., 1982). Labeled oligonucleotide was separated from free  $^{32}\text{P}$  with a  $\text{C}_{18}$  Sep-Pak column (Waters Associates, Milford, MA). Hybridization was performed in  $5\times$  SSPE,  $10\times$  Denhardt's, 250  $\mu\text{g}/\text{ml}$  tRNA, 7% SDS, and 10% dextran sulfate, for 18 h at  $37^{\circ}\text{C}$ . The membranes were then washed twice in  $1\times$  SSC + 0.1% SDS at  $20^{\circ}\text{C}$ .

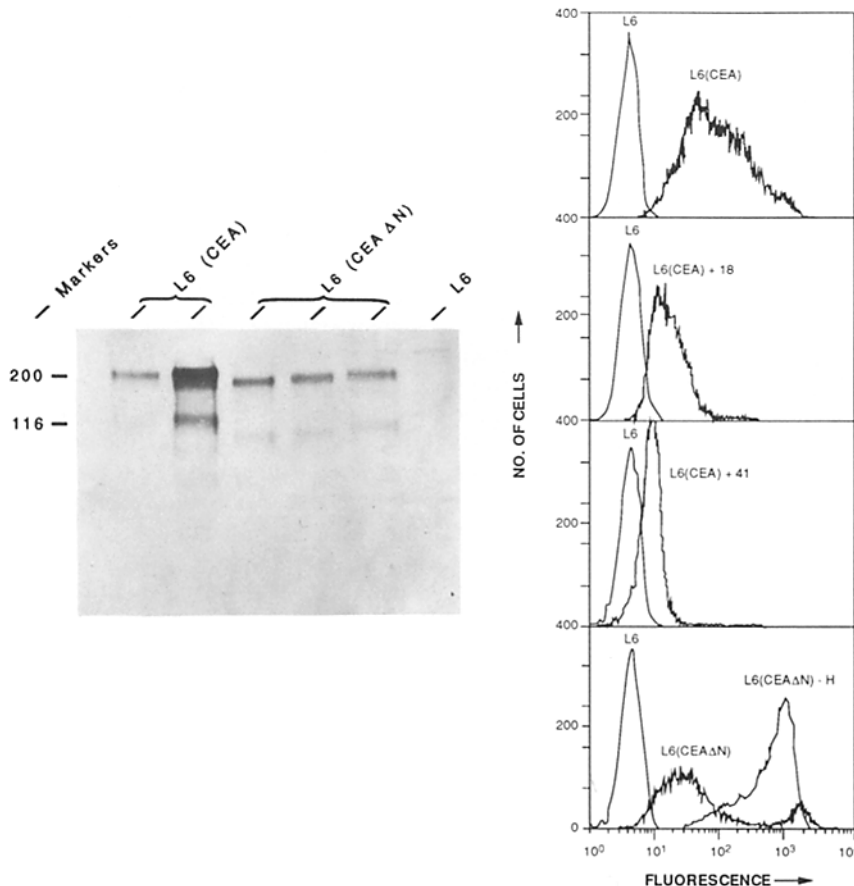
### Production of Bacterial Fusion Peptides

Constructs consisting of the 108-amino acid N domain and the 178-amino acid A1B1, A2B2, and A3B3 domains of CEA fused to the *E. coli* CTP:CMP-3-deoxy-manno-octulosonate cytidyltransferase (CKS) gene were expressed in *E. coli* and the products purified according to the protocol of Hass et al. (1991) as described previously (Zhou et al., 1993). After dialysis against D-MEM, the fusion peptides were added to differentiation medium to a final concentration of 1 mg/ml and incubation continued as indicated. In some cases medium was changed to medium containing fresh peptide after 2 days' incubation.

### Results

#### Effect of CEA Production on Fusion

L6 myoblasts were cotransfected with pSV2neo and with CEA, antisense CEA, or CEA $\Delta\text{N}$  cDNAs, and stable transfectant clones were selected. CEA $\Delta\text{N}$  contains a deletion of the last 75 amino acids of the 108-amino acid  $\text{NH}_2$ -terminal domain, retaining the processed leader sequence, and is completely defective in adhesion function when expressed on the surface of CHO cell transfectants (Oikawa et al., 1991; Zhou et al., 1993). Transfectant clones were picked and cell surface expression of CEA was assessed using polyclonal and monoclonal antibodies. CEA production in positive clones was between 40 and 800 ng CEA/mg protein of total cell lysate using a calibrated double monoclonal antibody assay. This same assay, however, did not detect the product expressed by the mutant CEA $\Delta\text{N}$  transfectants. A Western blot of electrophoretically separated total



**Figure 1.** Western blot and cytofluorimetric profiles of L6 transfectants. (Left) Samples of total cell lysate were electrophoresed on a 7.5% polyacrylamide gel and transferred to nitrocellulose paper. The blot was incubated with polyclonal rabbit anti-CEA antibody, and then with alkaline phosphatase-conjugated goat anti-rabbit Ig and developed with NBT-BCIP as described in Materials and Methods. The major bands at  $\sim 200$  kD in molecular weight represent fully glycosylated CEA, whereas the minor bands at  $\sim 120$  kD in molecular weight are due to partially glycosylated CEA, as shown for other transfectants (unpublished data). The very faint band at the top of the gel in the L6 lane was not reproducible. (Right) Cytofluorimetric profiles of parental L6 cells together with the L6 CEA transfectant L6-17, L6-17 after 18 and 41 passages without G418 selection, the L6 CEA $\Delta\text{N}$  transfectant L6-16 (Table I) and a high-producer population, L6(CEA $\Delta\text{N}$ )-H, sorted from the higher producers in the L6-16 CEA $\Delta\text{N}$  transfectant population by FACS; whole cells were stained using polyclonal anti-CEA rabbit antibody and analyzed as described in Materials and Methods. Fusion could not be induced in the L6-17 transfectant after 18 passages but was restored after 41 passages.

cell proteins from CEA and CEA $\Delta$ N transfectants probed with polyclonal CEA antiserum is shown in Fig. 1. No CEA bands could be detected in lysates of the parental L6 myoblasts. Two CEA transfectant clones, which produced 104 and 837 ng CEA/mg protein, showed prominent bands of the expected molecular weight for fully glycosylated CEA. Most of the data presented below was obtained using the clone, L6-17, producing 104 ng CEA/mg protein, although similar results were obtained with all transfectant clones producing more than 100 ng CEA/mg protein (Table I). The three CEA $\Delta$ N transfectant clones displayed intermediate levels of production of a protein with a predictably lower molecular weight (Fig. 1), which was shown by immunofluorescent staining with polyclonal anti-CEA antibody to appear on the surface of the cells (data not shown). Quantitative measures of the cell surface levels of CEA and CEA $\Delta$ N were provided by cytofluorimetric profiles of various transfectant clones stained with anti-CEA antibody, including a CEA $\Delta$ N high-producer population, L6(CEA $\Delta$ N)-H, obtained by FACS sorting of the second peak of the CEA $\Delta$ N transfectant (Fig. 1).

After reaching confluence and after the serum concentration of the growth medium is reduced from 10 to 2% 5 d after seeding the cultures, L6 myoblasts align and start to fuse into myotubes of multinucleated cells. A dramatic difference was observed in the ability of the L6 CEA transfectants to fuse; after 9 d in culture, these transfectants showed single cells which failed completely to align and fuse, whereas the parental cells and CEA $\Delta$ N transfectants showed extensive fusion (Fig. 2). The quantitation of this phenomenon is presented in Fig. 3. Seventy-nine percent of wild-type L6 myoblasts fused into myotubes by 9 d after plating; control CEA $\Delta$ N transfectants demonstrated a fusion index similar to that of the parental cells (82%). In contrast, CEA transfectants showed no fusion whatsoever (Fig. 3). A complete absence of fusion was also observed for two other CEA transfectant clones producing more than 100 ng CEA/mg protein whereas a reduced fusion index of 21% was seen for a clone producing 40 ng CEA/mg protein (Table I); all these clones were obtained from independent transfections. Ten independent control clones, including CEA $\Delta$ N, the CEA $\Delta$ N high-producer, CEA antisense, and pSV2neo (only) transfectants, fused normally (Table I and data not shown). The results are thus unlikely to be due to the selection of spontaneous L6 variants that lack fusion ability (Kaufman and Parks, 1977). To rule out this possibility entirely, the nonfusing CEA transfectant L6-17 was cultured in the absence of G418 selection for 43 passages, in order to lose production of CEA; at passage 18, cell surface production of CEA had fallen markedly (Fig. 1), yet fusion ability was essentially normal; at passage 37, when CEA production was only 0.5 ng/mg protein, fusion ability returned fully to previous nontransfected levels (79%) (Fig. 2 and Table I); L6-17 maintained under selection for the same number of passages, on the other hand, was still completely unable to fuse (data not shown). The fact that this reversion to full differentiation capacity required loss of virtually all CEA production also shows that very little CEA is required to block fusion. Lastly, growth rate differences were not involved since there was no significant difference in the rate of proliferation of the transfectants compared with the parental myoblasts; in addition, the cell volume of

Table I. Differentiation Parameters for L6 Transfectants

Clone	Transfected construct*	CEA production (ng/mg)	Fusion index	CPK $\ddagger$ (fold increase)
L6	—	0	0.81	11
L6-N	pSV2Neo	0	0.84	15
L6-27	CEA-antisense	0	0.78	18
L6-23	CEA-antisense	0	0.63	nd**
L6-16	CEA $\Delta$ N	>100 $\dagger$	0.82	19
L6-16-H	CEA $\Delta$ N	>300	0.50	nd
L6-18	CEA	0	0.83	13
L6-73	CEA	40	0.21	nd
L6-17	CEA	104	0	1
L6-71	CEA	212	0	nd
L6-82	CEA	837	0	1
L6-17-37(-) $\S$	CEA	0.5	0.79	10
L6-17-43(-) $\S$	CEA	0.2	0.76	10
L6-17+N $\parallel$	CEA	104	0.47	nd
L6-17+A3B3 $\parallel$	CEA	104	0.58	nd
L6-17+N+A3B3 $\parallel$	CEA	104	0.51	nd
L6-17+A1B1 $\parallel$	CEA	104	0.04	nd
L6-17+A2B2 $\parallel$	CEA	104	0.01	nd

\* All cell lines were cotransfected with pSV2Ne and expression constructs containing the indicated cDNA.

$\ddagger$  The fold increase in CPK activity was calculated relative to the activity of nonfusing L6 cells.

$\S$  The L6-17 CEA transfectant maintained in culture without selection with G-418 for 37 and 43 passages, resulting in a reduction in the original level (104 ng/mg) of CEA production.

$\parallel$  Fusion peptides CKS-N, CKS-A1B1, CKS-A2B2, and CKS-A3B3 made in bacteria and containing the N, A1B1, A2B2, or A3B3 domains of CEA were added to 1 mg/ml to transfectant cultures in differentiation medium and incubated for 4 d.

$\dagger$  Estimated from data shown in Fig. 1.

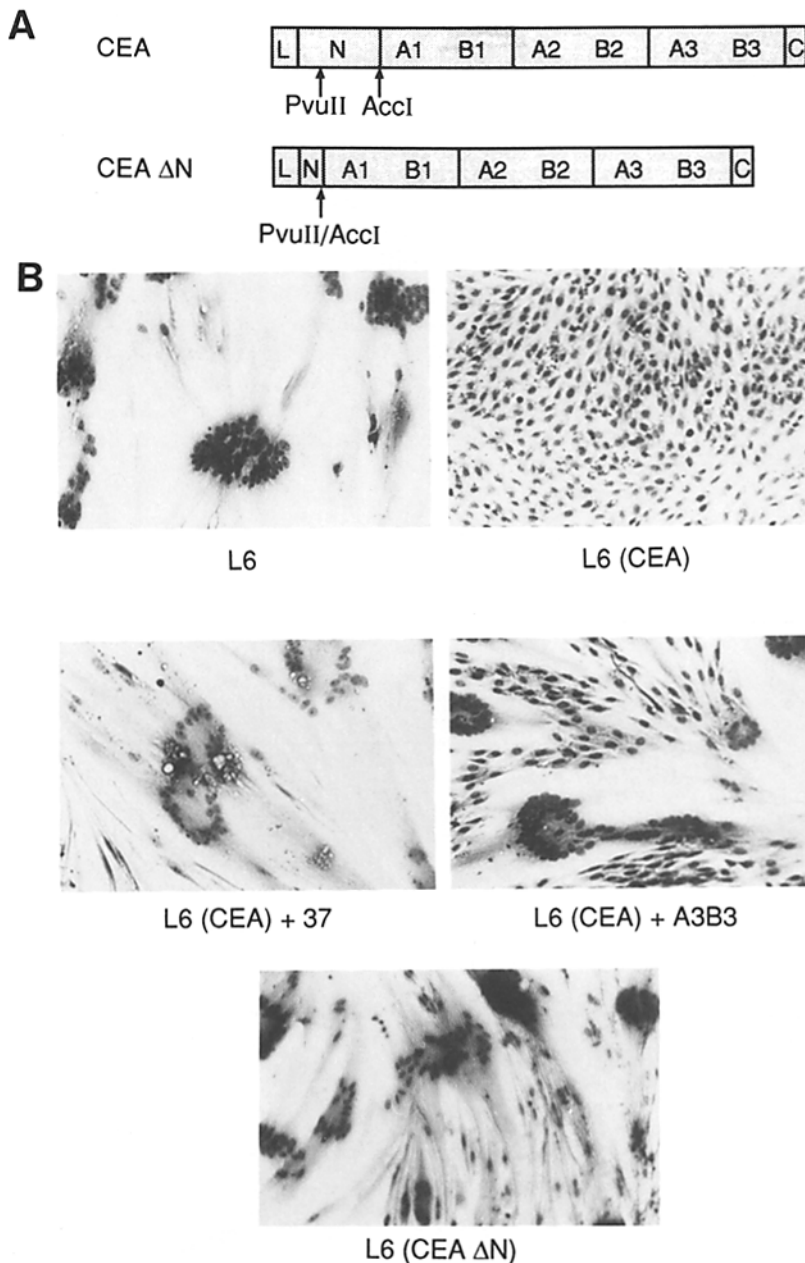
\*\* Not done.

proliferating CEA transfectant and parental myoblasts was identical (data not shown).

CEA-mediated adhesion requires a double-reciprocal interaction between the V-like N domains and the membrane-proximal C2-like A3B3 internal domains of antiparallel molecules on apposing cell surfaces; thus treatment with N domain or A3B3 domain peptides made in bacteria can inhibit intercellular adhesion completely, while A1B1 and A2B2 peptides are much less effective (Zhou et al., 1993). Treatment of nonfusing L6 CEA transfectants in differentiation medium with either or both of the N and A3B3 peptides was found to restore differentiation and fusion to 50–60% with approximately the same kinetics as the parental L6 cells when exposed to differentiation medium, whereas the A1B1 and A2B2 peptides were without effect (Table I and Fig. 2). The concentration of the N and A3B3 peptides required to restore fusion capacity to the L6 CEA transfectants (1 mg/ml) was similar to that required to block aggregation of CHO CEA transfectants in suspension (2 mg/ml) (Zhou et al., 1993). These results strongly implicate the necessity for adhesive interactions between CEA molecules on apposed cells to block L6 differentiation and fusion.

### Effect of CEA on Molecular Measures of Differentiation

Nonfusing myoblasts which still demonstrate all the molecu-



**Figure 2.** (A) Depiction of cDNAs and corresponding protein domains for CEA and CEA $\Delta$ N. (B) Morphology of parental L6 myoblasts, an L6 CEA transfectant clone after 37 passages to lose CEA production, the L6 CEA transfectant clone, treated with 1 mg/ml CEA A3B3 peptide for 4 d and the L6 CEA $\Delta$ N transfectant clone, under culture conditions promoting differentiation (see Materials and Methods). Cells were fixed in glutaraldehyde and stained with hematoxylin. (Magnification  $\times 200$ ).

lar changes required for differentiation have been described (Pinset and Whalen, 1984; Olson et al., 1987). To explore the possibility that the nonfusing phenotype in CEA transfectants was due to an uncoupling of fusion from differentiation, we examined the production of CPK, a muscle specific enzyme which is upregulated in differentiated myoblasts (Olson et al., 1983), myogenin, a helix-loop-helix key transcriptional regulator protein whose production is induced early in L6 myoblast differentiation (Wright et al., 1989), and  $\beta$ -actin which is downregulated in developing myotubes (Endo and Nadal-Ginard, 1987).

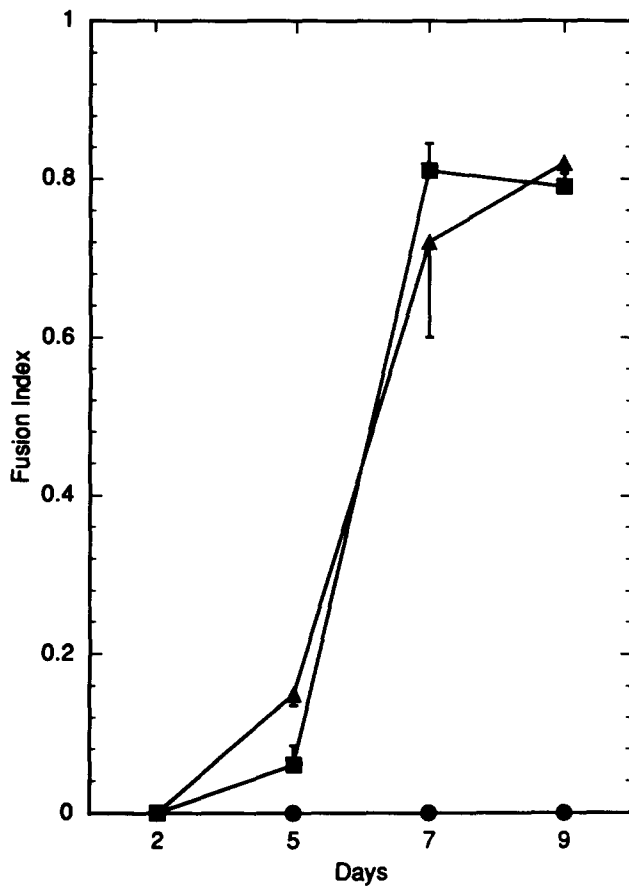
#### ***Creatine Phosphokinase***

CPK levels (Fig. 4, Table I) rose 10–20-fold after 7 d in cul-

ture in both L6 parental cells and in control CEA $\Delta$ N transfectants. In contrast, CEA transfectants showed no increase in CPK activity and remained at basal levels throughout. CEA antisense, pSV2neo, and L6-17 transfectants which had lost CEA expression due to prolonged passage without selection showed increases in CPK levels comparable to L6 parental cells (Table I). Thus, ectopic production of CEA completely blocks the normal induction of CPK with differentiation.

#### ***Myogenin***

Northern blot analysis of total cell RNA with myogenin cDNA as a probe (Fig. 5) demonstrated a dramatic rise in myogenin mRNA in the parental L6 myoblasts and control



**Figure 3.** Fusion index of L6 parental myoblasts and transfectants with time in culture. Parental L6 myoblasts (■) and CEA (●), or CEAΔN (▲), transfectants were plated at  $10^4$  cells/cm<sup>2</sup> and grown under fusion-promoting conditions. At the indicated intervals after plating, the cells were fixed, stained with hematoxylin, and the mean fusion index (nuclei in cells with >3 nuclei/total nuclei) was determined. Values shown are the mean of at least 3 determinations of >500 nuclei each. Error bars represent the standard error of the mean.

transfectants, starting 5 d after plating, i.e., before the large increase in the fusion index (Fig. 3). In contrast, the CEA-producing transfectant clones showed absolutely no increase in myogenin mRNA (Fig. 5). Since the induction of this central transcriptional regulator represents a key early event in the myoblast differentiation program, this result indicates that CEA intercepts the process at a relatively early stage.

### Actin

The same Northern blot used for the myogenin analysis was probed with  $\beta$ -actin cDNA. The predicted downregulation of actin mRNA in parental L6 myoblasts and control transfectants during fusion and differentiation was observed (Fig. 5). In contrast, the CEA L6 transfectant clone showed no reduction in the  $\beta$ -actin mRNA. The same blot rehybridized with a 24-mer probe specific for 18S RNA is shown to demonstrate that equal amounts of RNA were analyzed in each lane.

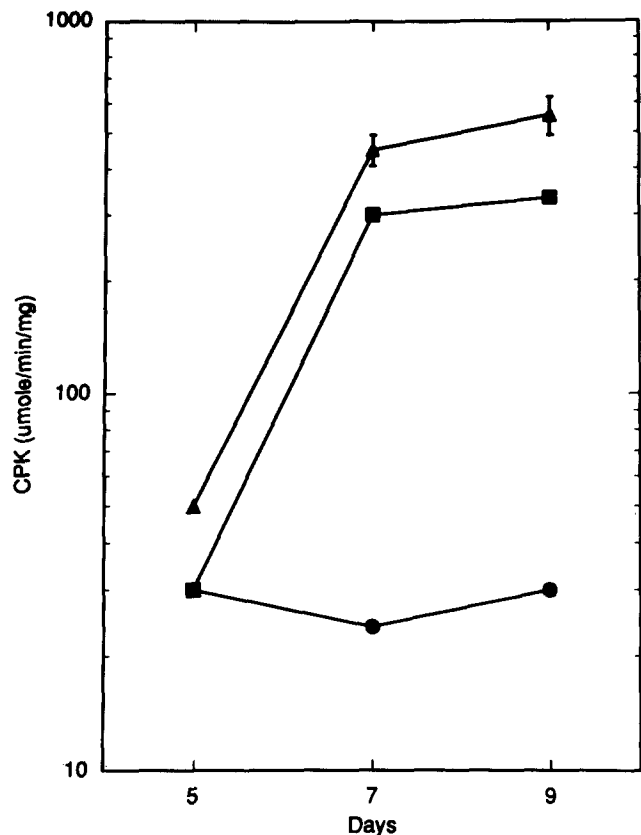
These results demonstrate that all three molecular aspects of myoblast differentiation, even relatively early events, were blocked by the production of CEA.

### Effect of CEA on Cellular Proliferative Potential

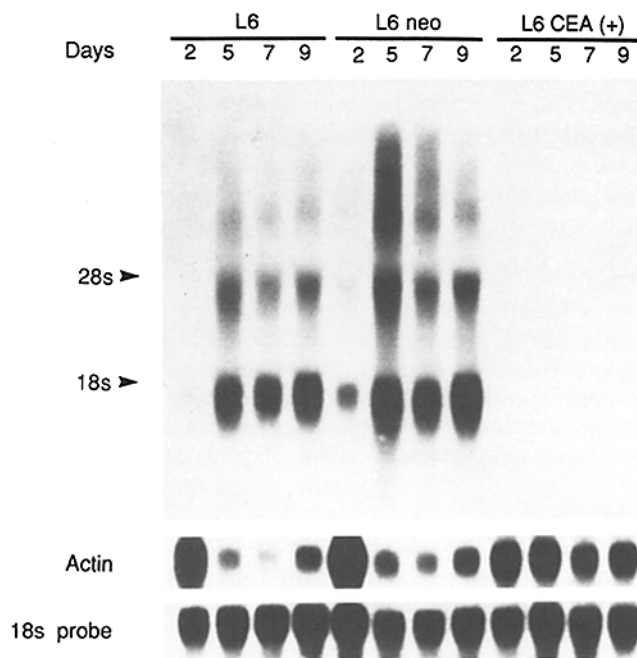
The relative plating efficiency for colony formation by L6 myoblasts and various transfectants at different times during the differentiation cycle was measured (Fig. 6). The plating efficiency of the parental myoblasts and CEAΔN transfectants declined markedly with time in culture and with kinetics which indicate that irreversible withdrawal from the cell cycle, as measured by this assay, precedes fusion and differentiation. The residual proliferative capacity of ~20% at day 9 reflects the proportion of nonfused cells. Comparable declines in plating efficiency were seen in control pSV2neo transfectants (data not shown). In contrast, CEA transfectant clones retained their proliferative capacity, maintaining a relative plating efficiency of ~75% until day 9. Thus the expression of CEA abrogated terminal differentiation and allowed cells to maintain their division potential.

### Discussion

The overexpression of CEA family members in many types of human tumors and their function *in vitro* as intercellular adhesion molecules raise the possibility that this correlation



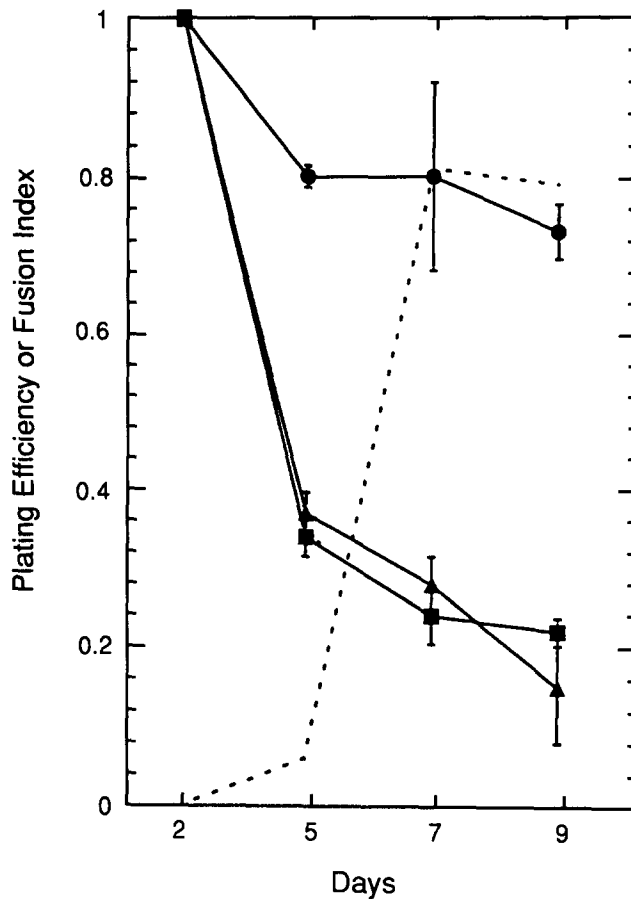
**Figure 4.** CPK production in L6 parental myoblasts and transfectants with time in culture. Parental L6 myoblasts (■) and CEA (●), or CEAΔN (▲), transfectants were plated and grown under conditions to promote fusion. Lysates were obtained at the indicated intervals and assayed for CPK production. Values shown are the means of three samples and are adjusted to the total protein content of each sample. Error bars represent standard errors of the mean and points with no apparent error bars have errors smaller than the symbols.



**Figure 5.** Myogenin and actin mRNA levels in L6 parental myoblasts and transfectants with time in culture. L6 myoblasts (L6), L6 transfected with PSV2neo alone (L6neo), or L6 transfected with CEA (L6 CEA+), were grown in culture under conditions to promote fusion. At the indicated intervals, cells were harvested, and total cell RNA was prepared. Aliquots of the RNA were resolved by electrophoresis, transferred to a nitrocellulose membrane, and hybridized successively with probes for myogenin and  $\beta$ -actin, under high stringency conditions. The same blot as rehybridized with a labeled 18S ribosomal RNA-specific oligomer to give an independent measure of the amount of RNA electrophoresed in each lane. The major band just below the 18s marker represents the myogenin transcript (Wright et al., 1989); the significance of the other bands is not known.

has a mechanistic basis. One such model suggested previously proposes a role for adhesion molecules in maintaining normal tissue architecture, with inappropriate expression in tumors leading to distortion in architecture and the disruption of cellular differentiation (Benchimol et al., 1989). In this model, cellular differentiation in multicomponent cellular systems is considered to depend on the accurate interplay of a number of recognition molecules on apposing cell surfaces; the introduction of a previously absent adhesion molecule, or a large change in the level of a previously existing one, is proposed to distort the fidelity of the process. To test this model, we have studied the effects of ectopic expression of CEA on differentiation in a model system, the rat L6 myoblast, and have found that expression of relatively low amounts of CEA on the surface of these cells results in a complete block in their ability to fuse and differentiate.

The possibility that CEA had uncoupled differentiation from fusion, as can occur, for example, when divalent cations are removed from the culture medium (Endo and Nadal-Ginard, 1987), is not the case because myoblast CEA transfectants did not show any of the molecular changes characteristic of differentiation, including the upregulation of creatine phosphokinase or myogenin mRNA levels or the downregulation of  $\beta$ -actin mRNA levels. Myogenin is a tran-



**Figure 6.** Plating efficiency of L6 parental myoblasts and transfectants with time in culture. Parental L6 myoblasts (■) and CEA (●), or CEAΔN (▲), transfectants were plated and grown under conditions to promote fusion. At the indicated intervals the mean plating efficiency (colonies/nucleus plated) was determined. Values shown are the mean of at least three determinations and are normalized to the plating efficiency at day 2. Error bars represent the standard error of the mean. The fusion index curve for L6 parental myoblasts (---) is shown for comparison.

scriptional regulator involved early in myogenic differentiation and believed to be pivotal in subsequent steps in the program (Wright et al., 1989) so that the complete inhibition of its normally dramatic upregulation indicates the interception by CEA of the differentiation program at a very early stage. Furthermore, CEA transfectants failed to withdraw from the cell cycle and maintained a high division potential. The activity of helix-loop-helix proteins such as MyoD and myogenin has been shown to depend on interaction with the active form of the cell growth cycle regulator, retinoblastoma protein (pRB), such that inactivation of pRB by phosphorylation or binding of viral proteins such as large T antigen can block myogenesis (Gu et al., 1993). It seems unlikely, however, that the effect of CEA on myogenesis is due to inactivation of pRB, since this would not itself explain the absence of myogenin mRNA. We are nonetheless currently investigating the status of pRB in the L6 CEA transfectants.

The results cannot be ascribed to the selection of nonfusing variants of L6. Thus, multiple independent CEA transfectant clones all failed to differentiate, whereas multiple

independent control transfectant clones all differentiated normally; in addition, full fusion capacity returned to a previously nonfusing CEA transfectant that had lost CEA production by extended culture without selection. Finally, fusion was not affected by the expression of very high levels of an adhesion-defective truncated CEA molecule with a deletion in the N domain and prompt fusion of L6 CEA transfectants was obtained after addition of CEA N domain or A3B3 domain peptides (Fig. 2), which inhibit CEA mediated intercellular adhesion. The latter, along with the failure of A1B1 and A2B2 domain peptides, which have much less effect on adhesion, to restore fusion, constitutes strong evidence that the proposed double reciprocal bonding of Ig V-like N to Ig C2-like A3B3 domains between antiparallel CEA molecules on apposed L6 cell surfaces (Zhou et al., 1993) is required for the antidifferentiation effect. Direct measurement of the adhesive effects of CEA and the antiadhesive effects of peptides in the L6 transfectants could not be achieved due to the presence of many other adhesion molecules in the parental L6 cells which gave a strong "background" intercellular adhesion. The rapid resumption of the differentiation program in L6 CEA transfectants maintained in differentiation medium when treated with N or A3B3 domain peptides (fusion begins within 1 day—unpublished results) indicates that these cells are primed for fusion and that other adhesion molecules required for fusion are not repressed. These results also show that the L6 CEA transfectants in confluent culture in differentiation medium are held in a state which can rapidly access either fusion or division.

Thus the entire program for differentiation, which L6 myoblasts normally undergo as a result of cell surface interactions involving ligand-receptors and other adhesion molecules such as cadherins and N-CAM, is abrogated by the presence on the surface of a new adhesion molecule, CEA. This effect is specific for CEA, since overexpression by transfection of myoblasts with cDNA coding for the GPI-linked form of another adhesion molecule of the immunoglobulin supergene family, N-CAM, which is itself positively involved in fusion, actually stimulated fusion by accelerating the process (Dickson et al., 1990). As mentioned above, the intercellular adhesive function of CEA is necessary for the effect. Our working hypothesis is that CEA interferes with the function of other adhesion molecules or signal transduction processes required for normal differentiation.

The interference envisioned could be extracellular ("ecto") by direct interaction of CEA with other cell surface molecules or intracellular ("endo") by interception of normal transduced molecular event sequences by a CEA-induced sequence. A special type of endo effect could arise from the fact that CEA is a heavily glycosylated protein; thus the possibility exists that its overexpression could saturate protein glycosylation pathways, thereby preventing the full maturation of cell surface glycoproteins required for the normal fusion and differentiation of L6 myoblasts, including other adhesion molecules. This explanation seems unlikely since the high-producer CEA $\Delta$ N transfectants, which expressed a slightly smaller but heavily glycosylated CEA analog on their cell surface at a model level of 100-fold more than the minimum amount of CEA required to block fusion (Fig. 1), had a normal fusing phenotype.

Considering ecto effects, CEA is a highly negatively charged molecule due to the terminal sialic acid residues on

its 28 N-linked sugar side chains. It could thus introduce a negative charge in the intermembrane space which could interfere with the adhesive function of other adhesion molecules. This hypothesis has been suggested for the antiadhesive effects of N-CAM seen in some situations, in this case because of its highly negatively charged specialized polysialic acid structure attached to a domain close to the cell membrane (Rutishauser et al., 1988). Again, if this is the mechanism of the CEA effect, it must also require CEA intermolecular adhesion since very high levels of the nonadhesive CEA $\Delta$ N molecule, with almost as many charged residues as CEA itself, on the cell surface allow L6 differentiation. Adhesion by CEA molecules could be required, however, since it would produce high local concentrations of charge at apposed regions of the membranes, so that this mechanism still seems plausible. Experiments are in progress to study directly a potential role of the terminal sialic acid residues in CEA.

Considering endo effects, the failure of the CEA transfectants to withdraw irreversibly from the cell cycle suggests that CEA production inhibits a terminal differentiation pathway which normally is induced after the cells reach confluence and experience a deficiency of mitogenic stimuli. This could be due to the stimulation by CEA of a competing pathway, thus representing an endo effect of CEA; BGP, a closely related family member with transmembrane and cytoplasmic domains, for example, has been suggested to be associated with protein tyrosine kinases in the cell membrane (Afar et al., 1992) and recent results have shown an association of CEA itself with *src*-family kinases (Draber, P., and C. P. Stanners, unpublished results). Alternatively, loss of proliferative capacity could simply be a terminal consequence of cell fusion so that blockage of fusion would naturally lead to retention of division capacity. This effect of CEA again requires an intact NH<sub>2</sub>-terminal domain, i.e., presumably its intercellular adhesion function, given that the CEA $\Delta$ N transfectants lost cell division potential normally.

Additional work will be required to elucidate the nature of the signals required for differentiation and loss of division potential which the CEA-CEA interactions on the cell surface distort or perhaps block. The generality of the phenomenon for the ectopic expression of other adhesion molecules in other differentiating systems also deserves investigation. Regardless of the mechanism involved, the failure to withdraw from the cell cycle and differentiate suggests that aberrant expression of CEA, a human tumor marker, could represent one step in the progression of a cell towards an overt malignant phenotype.

We thank Drs. P. C. Holland, O. Blaschuk, and Y. Pouliot for helpful discussions and Dr. Nicole Beauchemin for critical reading of the manuscript.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada. F. Eidelman was supported by a Medical Research Council of Canada Clinician Scientist award.

Received for publication 22 February 1993 and in revised form 6 July 1993.

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