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Oncogene *ect2* is related to regulators of small GTP-binding proteins

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We have developed an efficient expression cloning system that allows rapid isolation of complementary DNAs able to induce the transformed phenotype[1,2]. We searched for molecules expressed in epithelial cells and possessing transforming potential to fibroblasts, and cloned a cDNA for the normal receptor of a growth factor secreted by NIH/3T3 cells[3,4]. Here we report a second novel transforming gene, *ect2*. The isolated cDNA is activated by amino-terminal truncation of the normal product. The Ect2 protein has sequence similarity within a central core of 255 amino acids with the products of the breakpoint cluster gene, *bcr* (ref. 5), the yeast cell cycle gene, *CDC24* (ref. 6), and the *dbl* oncogene[7]. Each of these genes encodes regulatory molecules or effectors for Rho-like small GTP-binding proteins[8–10]. The baculovirus-expressed Ect2 protein could bind highly specifically to Rho and Rac proteins, whereas the *dbl* product showed broader binding specificity to Rho family proteins. Thus *ect2* is a new member of an expanding family, whose products have transforming properties and interact with Rho-like proteins of the Ras superfamily.

An *ect2*-transformed focus was induced after transfection of NIH/3T3 cells with a BALB/MK mouse keratinocyte expression cDNA library in λpCEV27 (ref. 2), and genomic DNA from the transformed cells was subjected to plasmid rescue[3]. A plasmid, designated p3N-11, had high-titred transforming activity on transfection of NIH/3T3 cells. The *ect2* foci exhibited an unusual stellate morphology containing both fusiform and rounded cells (Fig. 1*a*). A mass population of marker-selected *ect2*-transfected cells formed rapidly growing colonies at high efficiency (1–10%) in semi-solid agar medium, another *in vitro* characteristic of transformed cells. Subcutaneous inoculation of nude mice with 10^6 *ect2* cells led to palpable tumours in 100% of animals within 6 weeks, whereas marker-selected control NIH/3T3 cells failed to form any tumours, demonstrating that *ect2* transfectants exhibited the malignant phenotype.

Hybridization of the 2.8 kilobase (kb) *ect2* cDNA insert with poly(A)$^+$ RNA from BALB/MK cells revealed a single 4.0 kb transcript. The transcript was also detectable in kidney, liver, spleen and, at highest levels among the tissues analysed, in testis (Fig. 1*b*). By screening the BALB/MK cDNA library with the p3N-11 insert as probe, we isolated additional cDNA clones, whose sizes closely corresponded to the 4.0 kb transcript. Sequence analysis of one of these 4.0 kb cDNAs, designated CL7, revealed a long open reading frame of 2,214 base pairs (bp) encoding a predicted 84K protein (Fig. 2*a*). Comparison of the sequence of the p3N-11 clone with CL7 revealed that p3N-11 was truncated at its N terminus, beginning at amino-acid residue 212 in the complete *ect2* coding sequence. There was no match with any known gene in the GenBank and EMBL nucleotide and SWISS-PROT protein sequence databases. But

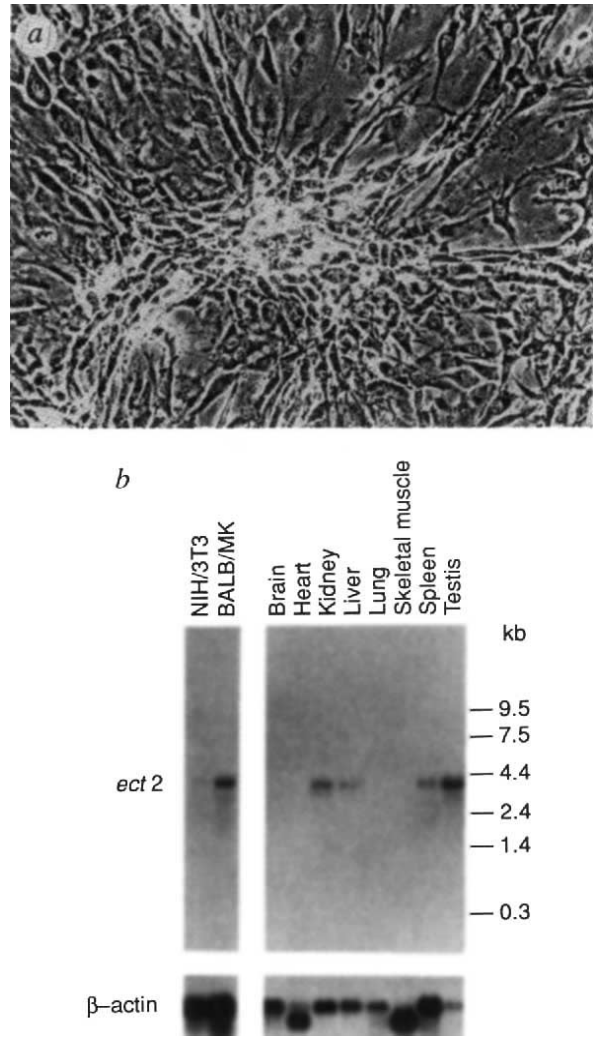

FIG. 1 Expression of the *ect2* gene. *a*, Cell morphology of the transformed foci induced by the *ect2* expression vector at 21 day post-transfection (×180). *b*, Northern analysis of poly(A)$^+$ RNAs from NIH/3T3 and BALB/MK cells and different mouse tissues. RNAs were fractionated, blotted and probed with (top) *ect2* cDNA insert from p3N-11 or (bottom) β-actin cDNA as described previously[3]. Molecular size markers are shown in kb.

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a

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   1  GAATCGGAGGCGGCAGCCTCTCTCGAGCCATGGCGGCGCCTGTGAGGGGGAGACTTAGTGTTTGAGAAGGATAAGCGAGGACTGCGCCGTCGTGGTTCCTTTTTAAAATCTCTGAGCGT
120  GGTGAGGACTGCGGGGTGAGTGGGGGACGGGAAGGGATTCGGGAACCCGGAACTGAGCACGGGCCGTGGCGACCAAGGCCGGCGCCCCCCGCCGCGCCTTCATCAAGTAGTGCAAACAATG
240  GCTGACGACAGCGTGTTACCATCTCCTTCTGAGATAACTAGCTTGGCAGACTCTTCAGTGTTTGATTCTAAAGTTGCTGAAATGTCCAAGGAAAACTTGTGCTTGGCGTCTACTTCAAAT
360  GTTGATGAAGAAATGCCGCAGTTGAAGCAAGAGTGATAATGGTCCAGGATGCTGGGAAACAAGAAGAACTTCTAAAGGCCTTAAAGGATTGTTGGGCCACCAGTTATACTCAACTGTGCA
480  CAAAGGGGAGAGCCCTTGCCGTTTTCCTGCCGGCCGCTGTATTGTACGAGTATGCTGAACCTGGTGCTCTGCTTCACTGGATTCAGGAAGAAGGAGGAGCTTGTCAAATTGGTGACGTTG
     1                                                                  M  L  N  L  V  L  C  F  T  G  F  R  K  K  E  E  L  V  K  L  V  T  L
600  GTTCATCATATGGGTGGAGTTATTCGAAAAGAGTGTAATTCCAAAGTAACACATCTGGTGGCAAATTGTACACAAGGCGAAAAATTTAGGGTTGCTGTGAGCCTGGGCACTCCAATTATG
 24  V  H  H  M  G  G  V  I  R  K  E  C  N  S  K  V  T  H  L  V  A  N  C  T  Q  G  E  K  F  R  V  A  V  S  L  G  T  P  I  M
720  AAGCCAGAATGGATTTATAAAGCGTGGGAAAGACGCAATGAACAGTGTTTCTGTGCAGCAGTTGATGACTTTAGAAATGAATTTAAAGTTCCTCCATTCCAAGATTGCATTTTAAGTTTC
 64  K  P  E  W  I  Y  K  A  W  E  R  R  N  E  Q  C  F  C  A  A  V  D  D  F  R  N  E  F  K  V  P  P  F  Q  D  C  I  L  S  F
840  CTGGGATTTTCAGATGAAGAGAAACATAGTATGGAAGAAATGACTGAAATGCAAGGAGGTAGCTATTTACCAGTTGGGGATGAAAGGTGCACTCACCTTATTGTTGAAGAGAATACAGTA
104  L  G  F  S  D  E  E  K  H  S  M  E  E  M  T  E  M  Q  G  G  S  Y  L  P  V  G  D  E  R  C  T  H  L  I  V  E  E  N  T  V
960  AAGGACCTTCCATTCGAACCTTCAAAGAAACTTTTGTTGTCAAGCAAGAGTGGTTCTGGGGAAGCATTCAGATGGACGCCTCGTGCAGGAGAGACTATGTATTTGTATGAAAAGGCTAAT
144  K  D  L  P  F  E  P  S  K  K  L  F  V  V  K  Q  E  W  F  W  G  S  I  Q  M  D  A  R  A  G  E  T  M  Y  L  Y  E  K  A  N
1080  ACTCCTGAGCTCAAGAAATCGGTGTCTCTGCTTTCTCTAAGTACTCCAAACAGCAACCGCGAAGAGACGGAGATTGAAAGAGACCCTGGCTCAGCTCTCCAGGGAGACTGACCTCTCTCCT
184  T  P  E  L  K  K  S  V  S  L  L  S  L  S  T  P  N  S  N  R  K  R  R  R  L  K  E  T  L  A  Q  L  S  R  E  T  D  L  S  P
1200  TTCCCTCCCCGGAAGCGGCCCTCAGCTGAGCACTCTCTTTCTATTGGCTCACTCCTGGATATCTCCAACACACCCGAGTCCAGCATCCACTATGGAGAAACACCGAAGTCCTGTGCTAAG
224  F  P  P  R  K  R  P  S  A  E  H  S  L  S  I  G  S  L  L  D  I  S  N  T  P  E  S  S  I  H  Y  G  E  T  P  K  S  C  A  K
1320  TCTTCTAGAAGTTCTACTCCAGTTCCTCCAAAGCAGTCAGCCAGGTGGCAGGTGGCGAAAGAGCTCTACCAGACTGAGAGCAATTATGTCAATATACTGGCCACAATCATTCAATTATTT
264  S  S  R  S  S  T  P  V  P  Q  S  A  R  W  Q  V  A  K  E  L  Y  Q  T  E  S  N  Y  V  N  I  L  A  T  I  I  Q  L  F
1440  CAGGTGCCCTTAGAAGAAGAAGGACAGCGTGGTGGGCCTATTCTTGCACCTGAGGAGATCAAGACTATTTTTGGGAGCATCCCAGATATCTTTGATGTGCATATGAAGATCAAGGATGAT
304  Q  V  P  L  E  E  E  G  Q  R  G  G  P  I  L  A  P  E  E  I  K  T  I  F  G  S  I  P  D  I  F  D  V  H  M  K  I  K  D  D
1560  CTTGAAGACCTTATTGCTAACTGGGATGAGAGCAGAAGTATTGGTGACATCTTTCTTAAATATGCAAAAGATTTGGTAAAAACCTACCCTCCGTTTGTAAACTTCTTTGAAATGAGCAAG
344  L  E  D  L  I  A  N  W  D  E  S  R  S  I  G  D  I  F  L  K  Y  A  K  D  L  V  K  T  Y  P  P  F  V  N  F  F  E  M  S  K
1680  GAAATGATCATTAAATGTGAAGAAACAAAAGCCCAGATTTCATGCTTTCCTCAAGATAAATCAAGCTAAACCAGAATGTGGACGACAGAGCCTTGTGGAACTTCTCATCCGGCCAGTGCAG
384  E  M  I  I  K  C  E  K  Q  K  P  R  F  H  A  F  L  K  I  N  Q  A  K  P  E  C  G  R  Q  S  L  V  E  L  L  I  R  P  V  Q
1800  AGGCTACCCAGTGTCGCTTTACTTTTAAACGATCTTAAAAAGCATACAGCTGATGAAAATCCAGACAAAAGCACTTTAGAAAAAGCTATTGGATCACTAAAAGAAGTAATGACACATATC
424  R  L  P  S  V  A  L  L  L  N  D  L  K  K  H  T  A  D  E  N  P  D  K  S  T  L  E  K  A  I  G  S  L  K  E  V  M  T  H  I
1920  AATGAGGATAAGAGAAAAACAGAAGCACAGAAGCAAATTTTTGATGTTGTTTATGAAGTAGATGGATGCCCAGCTAATCTCTTATCTTCTCATCGGAGCCTGGTGCAACGAGTGGAAACG
464  N  E  D  K  R  K  T  E  A  Q  K  Q  I  F  D  V  V  Y  E  V  D  G  C  P  A  N  L  L  S  S  H  R  S  L  V  Q  R  V  E  T
2040  GTTTCCCTTGGTGAGCACCCTTGCGACCGAGGAGAGCAAGTCACTCTCTTCCTCTTCAATGACTGCCTCGAGATAGCAAGAAAGCGGCACAAGGTTATTGGCACTTTTAGAAGTCCTCAC
504  V  S  L  G  E  H  P  C  D  R  G  E  Q  V  T  L  F  L  F  N  D  C  L  E  I  A  R  K  R  H  K  V  I  G  T  F  R  S  P  H
2160  GACCGCACCCGGCCCCCCGCTCTCTGAAGCACATTCATCTCATGCCTCTTCTCAGATTAAGAAGGTGCTGGACATCCGAGAGACAGAAGATTGTCACAATGCCTTTGCCTTGCTTGTA
544  D  R  T  R  P  P  A  S  L  K  H  I  H  L  M  P  L  S  Q  I  K  K  V  L  D  I  R  E  T  E  D  C  H  N  A  F  A  L  L  V
2280  AGGCCACCAACAGAACAGGCCAATGTACTGCTCAGCTTCCAGATGCAGTTCCAAAGGAGAGCTGGCTGAAGATGCTGTGCCGACATGTAGCCAACACCATTTCTAAGGCA
584  R  P  P  T  E  Q  A  N  V  L  L  S  F  Q  M  T  S  E  E  L  P  K  E  S  W  L  K  M  L  C  R  H  V  A  N  T  I  C  K  A
2400  GATGCTGAGAATCTTATGTATGTGGCTGATCCAGAATCCTTTGAAGTAAATACAAAAGATATGGATAGTACATTGAGTCGAGCATCTAGAGCAATAAAGAAGACTTCAAAAAAGGTTACA
624  D  A  E  N  L  M  Y  V  A  D  P  E  S  F  E  V  N  T  K  D  M  D  S  T  L  S  R  A  S  R  A  I  K  K  T  S  K  K  V  T
2520  AGGGCATTCTCTTTTTCCAAAACTCCCAAGAGAGCCCTGCGGATGGCTCTTTCATCATCCCATAGCTCAGAGGGAAGGAGTCCTCCGAGCAGTGGCAAGCTTGCTGTGAGCCGTCTGTCC
664  R  A  F  S  F  S  K  T  P  K  R  A  L  R  M  A  L  S  S  H  S  S  E  G  R  S  P  P  S  S  G  K  L  A  V  S  R  L  S
2640  AGCACATCATCCTTAGCAGGTATTCCATCTCCCTCCCTTGTCAGCCTCCCTTCTTTCTTTGAACGGAGAAGTCATACATTAAGTCGATCTACAACTCACTTGATATGAAACTTAAGTCCT
704  S  T  S  S  L  A  G  I  P  S  P  S  L  V  S  L  P  S  F  F  E  R  R  S  H  T  L  S  R  S  T  T  H  L  I  *
2760  GTGGGACCTATGCTTCAAACAAGACGCTGACTTAAGTGGTACTTTGAATTAGCACTTGGTAAAGTTGTAGAGAAGATTAGTAACACTAACCAACGCCGTTTTATTTTCTTGAAATACAATT
2880  TTCAAGATGGTCTATGTAAAAAAAAATATGGATTTTGATGTAATTTATCTTTATTTGAATCTCACATCTGAAGACCAGTGACTTAAGTTATTCCTGATAATCAGTAGCTTTGTAACCCTGG
3000  TACAGTAAACAGTTGGTGGGTGCAAAATACTATTGTGTATTTGTATAGTATCTTGAATTTTTGGAAGTACATATTTGTGTATCTCAATTTGTGCAAAGACTGAACAGCAATGCAACACTT
3120  GCTGAATAAAACCCTCTGATTAAATTCTAGAGGCTTGTTCAGTTCGATTGTTTGATGTACTTAACTGCGCTTTTGCAGTTTATTTTCTTTTAAAATAAGAGATCTAACACATGAATTTTA
3240  AAGGATACACCATTATTAACCATACAATTACGATCGTTTGGAATTTTCTTTTGCAAATTGAGATAAGAAGAGAAAGATTACAAAACATTGTATATGTTGCGAAGATAAGATATTAGTATG
3360  TACTTTATAATTGTTTCAGAGACTTTTATTTTATTTTATTTCTTATTCTACGACACTAGAAATCTTGATTTAGTTTTGAACTACTGTATTGAATTTATTTCAAAGGATAAGACCACGTG
3480  AAAACTGTGTTAAGAATTGCTTCTGTTCTTTGTACAGTTCTGAAGTTGTCTTCAGTTTTGCTAATCGCCTGTGAAAAGCATAGATATAATGTGTGGTGAACTTTTTATTGTGGTCATATC
3600  ATTAAAATAAGATTGGAAATTTATTGTCAAAAATTTTGTGAATCTGCCATTATCTTCTACTGCAATATTTCTTTTAGTCAGTAATACAGAATGACTGGGCTACTATGGCCAAGAATATTT
3720  TATTTCTTACACGTTCAGAGGAGTGTTACAGGTCTCCTTGTTACATAATTTTCAATAATCTGCTTTAATAGTTTAACTGACTGGATTTTTTTCTATACCATGTATGTACCAATTCACAGG
3840  TTAGTAAATGACCTTGTTTTGCTATATTTTAAAAGCAGTTATATTAGCAAGAACTTTGTAAATAAATAGCTTTAAAATACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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          .  =..=.  .=*.  *=   *...= =  ....              =  .....  ..*..
bcr   501 W-VLSGILASETTLSHLEALL-LPMKPLKAAATTSQ--PVLTSQQIETIFFK
ect2  166 WQVAKELYQTESNFYVNILATIIQLFQVPLEEEGQRGG--PILAPEEIKTIFGS
dbl   497 NHYLNELIQTERVYVRELYTVL-LGYRAEMDNPEMFDLMPPPLRNKKDILFGN
CDC24 162 VKIIKEFVATERKIVHDLE-IL-DKYRQQLLDSN------LITSEELYMLFPN
          ..*=.===.**. **..*.=** =..==...=*=  ..  =====*==..**=.

          .  .....=  .....*=  .  .  .=.*..*.. .  ..   *  ...  .
bcr   550 VEELYEIHKE-SYDGLFPRVQQWSHQQRVGDLFQKLASQLG-VYRAFVDNYGV
ect2  217 IPDIFDVHMKIK-DDLEDLIANWDESRSIGDIFLKYAKDLVKTTPPFVNFFEM
dbl   549 MAEIYEFHNDIFLSSLENCAH---APERVGPCFLERKDDFQ-MYAKYCQNKPR
CDC24 207 LGDAIDFQRR-FLISLETNALVEPSKQRIGALFMHSKHFFK-LYEPWSIGQNA
          ***==*.=......=*=.  . .....==**.=*==  ..=.  =*.=*.=....

          .  =.  =.    ..=  =  ..  =  .  .  ..     ...*  ..*.=**=*....
bcr   601 AMEMAEKCCQANAQFAEISENLRARSNKDAKDPTTKNSLETLLYKPVDPVTRS
ect2  269 SKEMIIKCEKQKPRFHAF---LKI--N-QAKPECGRQSLVELLIRPVQRLPSV
dbl   598 SETIWRKYSEC-AFFQECQRKL----------KHRLRLDSYLLKPVQRITKY
CDC24 258 AIEFLSSTLHK-MHVDESQRFI-T---------NNKLELQSFLYKPVQRLCRY
          *.==.  =. .  =.=.= .. *..  . ...   .*..*== *.******==.

           *==..*==..  = ...  .*..*=....  .  ==*=  . ..    ....
bcr   654 TLVLHDLLKHTPASHPDHPLLQDALRISQNFLSSINEEITPRRQSMTVKKGEH
ect2  316 ALLLNDLKKHTADENPDKSTLEKAIGSLKEVMTHINEDKKRTEAQKQIFDVVY
dbl   639 QLLLKELLKYSKDCE-GSALLKKALDAMLDLLKSVNDSMHQIAINGYIGNLNE
CDC24 300 PLLVKELLAESSDDN-NTKELEAALDISKNIARSINENQRRTENHQVVKKLYG
          =***=**==.*==.=.=.=.*=.**....*==.=***=.=.....  *..=.

          ...  .  .  .  .  .  . =.. .  .  .  .     ... .. .. ...
bcr   707 RQLLKDSFMVELVEGARKLRH-VFLFTDLLLCTKLKKQSGGKTQQYDCKWYIP
ect2  369 EVDGCPANLLESHRSLVQRVETVSLGEHPCDRGEQVTLFLFNDCLEI-ARKRH
dbl   691 LG---KMIMQGGFSVWIGHKK-GATKMKDLARFKPMQRHLFLYEKAIVFCARR
CDC24 352 RVLNWKGYRISKFGEELYFDK-VFISTTNSSSEPEREFEVYLFEKITILFSEV
          ...  .=.===.....= ..= =.=.............  ==..=. .= ....
```

FIG. 2 *a*, Nucleotide and predicted amino-acid sequence of the *ect2* cDNA. The longest *ect2* cDNA insert (CL7) was used to derermine the nucleotide sequence. Predicted amino-acid sequence is shown from the first ATG codon in the open reading frame. Nucleotide and amino-acid numbers are shown at the left. Polyadenylation signals are underlined. Complete sequences of CL12 and p3N-11 were also determined and they were identical to CL7 in the overlapping regions. *b*, Sequence comparison of the dbl-homology (DH) domains of the four proteins. Amino acids of CDC24 are numbered according to the revised sequence[23]. Gaps were imposed by dashes to give the maximum homology. The amino-acid residues identical to that of the Ect2 protein are shadowed. Sequence similarity between all four proteins are shown at the top: Asterisk, equals sign and dot indicate that the residue is common in four, three and two proteins, respectively. The bottom line shows a similar analysis but conservative amino-acid changes are taken into account. The conservative amino acids are grouped as follows: D, E, N, Q; H, K, R; A, G, P, S, T; I, L, M, V; F, W, Y; C (single-letter code). Amino-acid numbers are at the left.

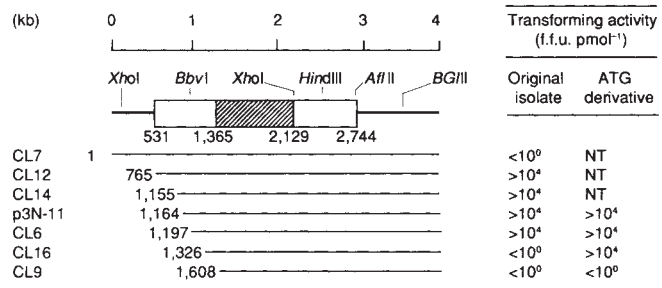


FIG. 3 Schematic representation of the *ect2* cDNA clones and their transforming activities. Untranslated regions are shown by thick lines and the coding sequence is shown by a box. Hatched portion indicates the DH domain. Positions of nucleotides that delimit the domains are shown under the coding region box. The region carried by each clone is shown by a line and the nucleotide position of each 5′-end in the *ect2* CL7 sequence is indicated (see Fig. 2*a*). f.f.u., Focus-forming units; NT, not tested.
METHODS. The first *ect2* plasmid, p3N-11, was rescued from transformed cells of the *ect2* focus. Other cDNA clones were isolated by screening of the BALB/MK library with p3N-11 cDNA insert as a probe. Plasmids with the same structures as p3N-11 and CL6 were also identified in two other *ect2* foci induced by the BALB/MK cDNA library. To add a consensus translation initiation site at the 5′-end of the truncated cDNA clones, oligonucleotides were inserted between the *Bam*HI site (in the vector) and the *Nru*I site (in the *Sfi*I adaptor[2]) located upstream from the cDNA sequence. The resulting plasmids (ATG derivatives) were sequenced to confirm the presence of the ATG codon in-frame with the truncated *ect2* coding sequence. The ATG oligonucleotides had following sequences: 5′-GATCCTAGGCCACC*ATG*-GAAATCG-3′, 5′-CGATTTCCATGGTGGCCTAG-3′. Transformed foci were scored 21 days after transfection. In all of the transfection experiments, more than 10^4 of G-418-resistant colonies were formed per pmol of each plasmid DNA. Two other 4-kb *ect2* clones similar to CL7 did not show detectable transforming activity.

the predicted Ect2 protein contained a central domain consisting of 255 residues, which had sequence similarity with analogously located domains within the *bcr*, *CDC24*, and *dbl* gene products[11] (Fig. 2*b*). The core region of dbl-homology (DH) domain (amino acids 281–465) of *ect2* showed 31%, 22% and 24% identity, respectively, with those of Bcr, CDC24 and Dbl proteins. A chromosomal rearrangement of *bcr* and *abl* is involved in the activation of the *bcr-abl* oncogene in chronic myelogenous leukaemia (CML)[5]. *CDC24* is a yeast cell cycle gene associated with defective budding[6], whereas NIH/3T3 transfection of a human tumour DNA led to identification of the *dbl* transforming gene[7]. No other similarities were detected between the *ect2* product and any of these molecules.

To investigate the mechanism involved in activation of trans-

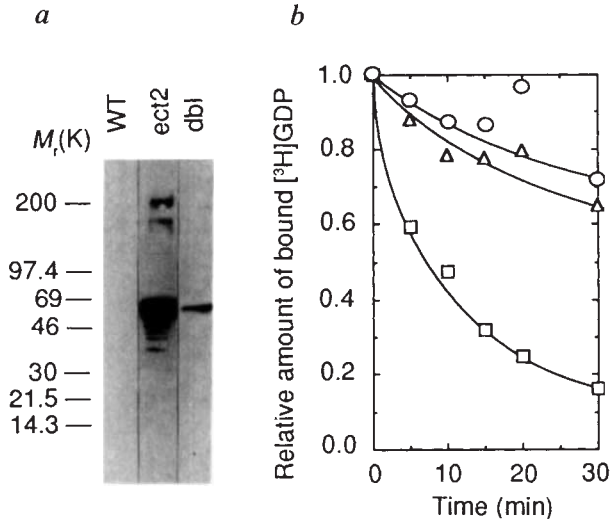
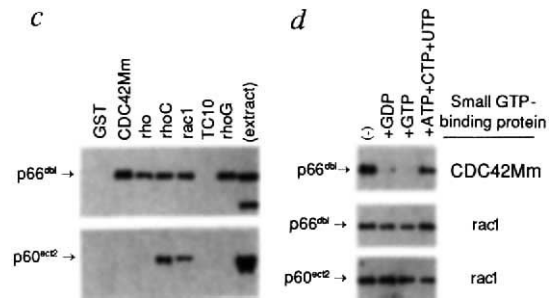


FIG. 4 Expression and functional analysis of the Ect2 protein. *a*, Production of the FLAG epitope-tagged Ect2 protein in insect cells. Soluble fractions (20 μl each) of *S. frugiperda* (Sf9) cells infected with wild-type (WT), FLAG-Ect2-expressing (ect2), or Dbl-expressing (dbl) baculovirus were analysed by immunoblotting using anti-FLAG M2 monoclonal antibody (IBI, New Haven) for WT and ect2, or anti-dbl antisera raised against a synthetic peptide[10] for dbl. The bands were visualized by ECL detection system (Amersham). Locations of molecular mass markers are at the left. *b*, Effects of the Sf9 cell extracts containing Ect2 protein on [³H]GDP dissociation from the murine CDC42 protein. Extracts (8 μl) of Sf9 cells infected with the wild-type virus (circles), FLAG-Ect2 virus (triangles), or Dbl virus (squares) were added to incubations (32 μl) containing the murine CDC42 protein (CDC42Mm) and the time courses for the dissociation of [³H]GDP (Amersham; 12.6 Ci mmol^{-1}) from the GTP-binding protein were measured as described[10]. The assay was done several times under similar conditions and a typical result is shown. *c*, Binding of Dbl and Ect2 proteins to Rho family small GTP-binding proteins. Binding assays were done as described[20]. The baculovirus-infected Sf9 cell extracts were dialysed and precleared with GST-beads. Aliquots (0.1 ml) were mixed with GST-beads or GST-Rho family protein beads (indicated on top) to precipitate the Dbl or Ect2 protein. The samples, in addition to the precleared lysates (10 μl each), were analysed as in *a*, except that affinity-purified anti-dbl rabbit polyclonal antibodies (Santa Cruz Biotechnology, CA) were used to detect Dbl. The lower band in the Dbl extract seems to be a degradation product of $p66^{dbl}$. *d*, Effect of



the presence of guanine nucleotides on binding. Binding assays were done in the absence (leftmost lanes) or presence (other lanes) of 0.5 mM of GDP, GTP, or mixture of ATP, CTP and UTP, as indicated, and processed as in *c*. The Rho family proteins used to precipitate Ect2 or Dbl are shown at the right.
METHODS. Oligonucleotides were inserted at the 5′-end of the *ect2* cDNA insert of p3N-11 (see Fig. 3) to add the FLAG epitope of 8 amino acids[24] followed by an ATG codon at the N terminus of the protein. The ATG-FLAG-*ect2* plasmid showed a similar focus formation efficiency on NIH/3T3 cells as p3N-11, indicating that the fusion protein is functionally active. The ATG-FLAG-*ect2* insert was then subcloned in a baculovirus expression plasmid pVL941, and the DNA was used to transfect Sf9 cells with linearized baculovirus DNA using BaculoGold transfection kit (PharMingen, San Diego, CA). The Sf9 cells were infected with the wild-type or *ect2*-recombinant virus and collected 60 h after infection. The *dbl*-recombinant virus-infected cells has been described[10]. These infected cells were sonicated in an extraction buffer[10] and the supernatants obtained after centrifugation for 15 min at 4 °C were used in all the experiments. The small GTP-binding proteins were cloned by polymerase chain reaction (PCR) in pCEV30G (T.M., unpublished data), a bacterial expression plasmid based on the T7 promoter[25], to fuse the proteins in frame with GST. The entire sequence of each insert was determined. The mouse *CDC42* clone (*CDC42Mm*) was 95% identical to the human clone (*CDC42Hs*)[13,14] in nucleotide level, and their amino-acid sequences were completely identical. Other clones were isolated from human cDNA libraries and their sequences were as reported except follows. Nucleotides A, C, G and A in positions 526, 564, 567 and 570 in the published *rhoG* sequence[26] were found to be G, G, C and G, respectively in our clone. Our sequence agrees to the hamster *rhoG* cDNA sequence[26] in these positions. The nucleotide position 452 of the published *rho* sequence[27] was found to be T in our sequence. These proteins were expressed[25] and purified[28] as described. The CDC42Mm protein was prepared by thrombin cleavage of the GST-CDC42Mm fusion protein followed by removal of the GST moiety[28].

forming activity, we compared biological activity of p3N-11 with the full-length *ect*2 cDNA as well as other clones isolated by screening the BALB/MK cDNA library. Figure 3 shows that the 4.0 kb *ect*2 cDNA exhibited no detectable transforming activity, whereas the truncated clones (CL12, CL14, p3N-11 and CL6) induced greater than 10^4 focus-forming units per pmol under the same assay conditions. These results indicated that truncation of its N-terminal region was involved in the activation of *ect*2 transforming function.

Analysis of CL16 and CL9 revealed the extent to which N-terminal truncation could be done without loss of transforming function. Although neither of these clones showed detectable biological activity, both lacked a strong consensus translation initiator. Therefore we inserted oligonucleotides containing a consensus initiator at the 5′ ends of these clones as well as p3N-11 and CL6. The ATG-derivative of CL16 exhibited high-titred transforming activity, similar to that of p3N-11. In contrast, CL9 remained nontransforming. These results suggested that deletion of an additional 96 residues including the first 81 amino acids of the conserved domain was sufficient to inactivate *ect*2 biological function.

The central core of *ect*2 gene product has significant homology with the Bcr, CDC24 and Dbl proteins. These proteins have been implicated as regulators or effectors of Rho-like products of the Ras superfamily of small GTP-binding proteins. A stretch of 29 amino acids near the C terminus of the *bcr* protein shows similarity to a purified GTPase activating protein (GAP) for $p21^{rho}$ and another GAP, *n*-chimaerin[8]. Moreover, the bacterially expressed Bcr protein C terminus exhibits *in vitro* GAP activity for $p21^{rac}$ (ref. 8). The defect in yeast budding associated with *cdc24* is shared by another mutant, *cdc42* (ref. 12), whose human homologue *CDC42Hs* encodes a Rho-related small GTP-binding protein[13,14]. The *cdc24* lesion can be suppressed by overexpression of *CDC42*, implying that *CDC24* may be a regulator of this GTP-binding protein[9]. The baculovirus-infected insect cell extract containing *dbl* oncogene product catalyses GDP/GTP exchange on CDC42Hs *in vitro*[10]. To test whether Ect2 protein can also act as an exchange factor towards CDC42, we cloned the murine CDC42 cDNA and purified the protein. The transforming Ect2 protein was expressed in a baculovirus expression system[15] (Fig. 4*a*), and the extract of the virus-infected cells was used to assay for guanine nucleotide exchange activity. The Ect2 protein-containing extract did not catalyse GDP/GTP exchange on the purified CDC42Mm protein, whereas the Dbl protein-containing extract possessed this activity, although it was much lower than the reported activity on the CDC42Hs protein purified from human platelet[10] (Fig. 4*b*). We also could not detect exchange activity in the Ect2 extract on Rho and Rac1 proteins expressed as glutathione *S*-transferase (GST) fusion proteins (data not shown). These results suggested that the Ect2 protein alone had no catalytic activity as an exchange factor on the Rho family small GTP-binding proteins under these conditions. The Ect2 protein may have exchange activity for other small GTP-binding proteins, or possibly modulate the activity of small GTP-binding proteins by an unknown mechanism.

A small GTP-binding protein, Ran, associates with its cognate exchange factor, RCC, and this complex dissociates in the presence of excess GDP or GTP[16], suggesting that regulators of small GTP-binding proteins can be identified by their specific binding. To test whether the Ect2 protein can interact with Rho family small GTP-binding proteins, we expressed and purified several more Rho family small GTP-binding proteins in addition to CDC42Mm as GST fusion proteins and examined specific binding to the baculovirus-expressed Ect2. The Ect2 protein showed specific binding to RhoC and Rac1 and, with less efficiency, to Rho (Fig. 4*c*). In contrast, Dbl protein showed specific binding to all of these Rho family proteins except TC10. The defective binding of Dbl protein to TC10 may be explained by possible N-terminal truncation of the TC10 protein, because this protein was expressed from the third methionine codon of the reported cDNA sequence[17]. In the presence of excess GDP or GTP, the specific binding of Dbl protein to CDC42Mm was drastically reduced, whreas the presence of other nucleotides had little effect (Fig. 4*d*). These binding properties were consistent to the exchange activity of Dbl protein on CDC42Mm. In contrast to CDC42Mm, Rac1 could form a complex with either Dbl or Ect2 protein (Fig. 4*c*). These complexes were detected even in the presence of guanine nucleotides (Fig. 4*d*). Because only a weak GDP/GTP exchange activity of Dbl protein on Rac1 was reported[10] and we could not detect exchange activity of Ect2 protein on Rac1, this guanine nucleotide-insensitive binding may be attributed to another function rather than guanine nucleotide exchange. Detailed mapping is needed to find specific binding sites of these proteins, but DH domains may be involved in binding to Rho family proteins. Our findings strongly suggest that Ect2 protein can interact with and may modulate the activity of Rho and Rac proteins and/or possibly closely related unknown small GTP-binding proteins.

Although members of the Bcr/CDC24/Dbl protein family share common features, each has different structural motifs and functional activities. For example, the CDC24 protein can bind Ca^{2+} ions[18], whereas the *bcr* product has serine/theronine-kinase activity[19] as well as a motif which allows binding to the SH2 domain of the *abl* oncogene product[20]. Recently two other molecules with DH domains were reported; Vav, an oncogene product which contains SH2 and SH3 domains[21], and Ras-GRF, a GDP/GTP exchange factor for Ras proteins[22]. Further characterization of *ect*2 and others of this family should help in elucidating signal transduction mediated by the small GTP-binding proteins as well as aberrations in these pathways associated with oncogenic transformation. □

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