

Fig. 4. Depletion of ET-like immunoreactive products in the posterior lobe of the rat pituitary gland after water deprivation. Male Wistar rats weighing about 300 g were used. (A) Numerous dot-like immunoreactive products are observed in the posterior pituitary supplied with food and water ad libitum. (B) Four days after water deprivation. Body weight decreased from 290 g to 230 g (21% loss). ET-like immunoreactive products in the posterior pituitary are largely depleted. All animals were perfused just before noon. Cryostat sections (10 μ m thick) were processed as described in Fig. 1. Bars, 150 μ m.

with a sensitive sandwich-type enzyme immunoassay was reported to be around 1.59 pg/ml (19), suggesting the presence of the circulating pool of ET. These data are consistent with the possibility that ET is a circulating hormone. Although the contribution of ET in the posterior pituitary system to the circulating level of ET remains unclear, our results suggest that ET has a role in neurosecretion.

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6. [Arg¹]ET(15–21) was synthesized with an automatic peptide synthesizer (Applied Biosystems Model 430A) and was coupled to KLH with *m*-maleimidobenzoyl-hydroxysuccinimide ester (5). Male New Zealand rabbits were immunized by subcutaneous injection with ETc-coupled KLH in complete Freund's adjuvant (300 μ g). Booster injections with the same amount of the antigen were given six times at 2-week intervals. The ETc antiserum was obtained 10 days after the last booster injection. The titer and cross-reactivity of ETc antiserum were evaluated by RIA. ETc antiserum failed to cross-react with the following peptides at more than 10⁴-fold molar excess: [Arg⁸]vasopressin, [Lys⁸]vasopressin, oxytocin, vasopressin-associated neurophysin (human), oxytocin-associated neurophysin (human), [Met⁵]enkephalin, [Leu⁵]enkephalin, dynorphin A, corticotropin-releasing factor, cholecystokinin octapeptide, neuropeptide Y, angiotensin I (human), and porcine big ET-1.
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10. ET-positive axons from PVN and SON, which passed through the fiber layer of the median eminence and projected to the posterior pituitary, showed varicosities and localized swelling resembling Herring's bodies. In the posterior pituitary, an accumulation of dot-like immunoreactive products for ET was concentrated around the small vessels.
11. For RIA with ETc antiserum, standard ET-1 or unknown samples in 100 μ l of RIA buffer [0.05M PBS (pH 7.4), 0.1% bovine serum albumin, 0.1% Triton X-100, 0.15M NaCl, 0.025M EDTA \cdot 2 Na, 0.05% NaN₃, and Trasylol (500 kallikrein inactivator units per milliliter)] were previously incubated with diluted ETc antiserum (200 μ l, 1:21,250) for 12 hours at 4°C. Then each standard or sample was incubated with ¹²⁵I-labeled ET-1 (50 μ l) (approximately 15,000 cpm) for 24 hours and was then incubated with diluted goat antibodies to rabbit immunoglobulin G (IgG) (500 μ l, 1:200) for 30 min at 4°C. After centrifugation, the radioactivity in each precipitate was determined. The median inhibitory concentration (IC₅₀) of tracer binding by ET-1 was observed at 600 fmol per tube. Cross-reactivity of this antiserum with ET-2 or ET-3 is almost the same as with ET-1.
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Mutations of the Adenylyl Cyclase Gene That Block RAS Function in *Saccharomyces cerevisiae*

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The interaction between RAS proteins and adenylyl cyclase was studied by using dominant interfering mutations of adenylyl cyclase from the yeast *Saccharomyces cerevisiae*. RAS proteins activate adenylyl cyclase in this organism. A plasmid expressing a catalytically inactive adenylyl cyclase was found to interfere dominantly with this activation. The interfering region mapped to the leucine-rich repeat region of adenylyl cyclase, which is homologous to domains present in several other proteins and is thought to participate in protein-protein interactions.

DOMINANT INTERFERING MUTATIONS can be useful for investigating interactions between components of signal transduction systems (1). Such a mutation can inhibit signal outputs by causing the production of a partially functional protein. The protein is functional in that it binds to an appropriate target, but nonfunctional in that the binding event is not productive. Thus, the mutant molecule sequesters its partner and prevents it from

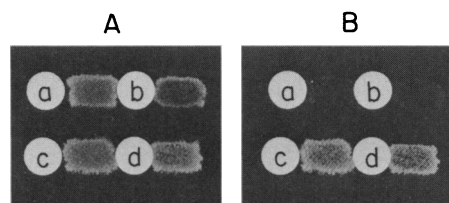
interacting with other components of the system. The net result is a diminished output from the signaling system.

RAS proteins activate adenylyl cyclase in *Saccharomyces cerevisiae* (2, 3), and several dominant interfering mutations of RAS have been found (4–7). One of these mutant proteins (7) appears to act by sequestering an “upstream” component, the *CDC25* gene product, which is thought to be an activator of yeast RAS. Feig and Cooper (5) isolated a similar mammalian *ras* mutation that appears to interfere with activation of mammalian Ras. Michaeli *et al.* (6) demonstrated that mutant RAS proteins that fail to translocate from the cytoplasm to the membrane also

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Fig. 1. Suppression of heat-shock sensitivity in a *RAS2^{val19}* strain by truncated adenylyl cyclase. Standard yeast genetic methods (26) were used to introduce plasmids into TK161-R2V, a strain of *S. cerevisiae* that carries the *RAS2^{val19}* allele of *RAS2* (2, 8). Cells were then patched and grown for 2 days on synthetic plates lacking leucine to maintain the plasmids that encode the *LEU2* marker (26). Two replica plates were made. One plate was kept at room temperature throughout the procedure (A) and the other was subjected to heat shock for 10 min at 55°C (B). Both plates were then incubated at 30°C for 2 days and photographed. Plasmids introduced into the yeast were as follows: (a) YEp13, a yeast vector that contains *LEU2*; (b) pYCYR, a vector built from YEp13 that expresses the full-length 2026-amino



acid adenylyl cyclase under the control of the *ADH-I* promoter (27); (c) pYCYRfs, a vector built from pYCYR that contains a frameshift mutation at the *Nco I* site located at the codon for amino acid 1608 (27); and (d) pYCAΔ5fs, a vector built from pYCYRfs that contains deletions of the coding sequences for amino acids 1 to 605 and 1302 to 1608.

interfere with RAS function in yeast; such mutants appear to sequester a “down-stream” component of the RAS pathway. We now describe dominant interfering mutations of the adenylyl cyclase gene *CYR1*.

To genetically screen for interfering forms of adenylyl cyclase, we made use of the heat shock-sensitive phenotype of cells containing the yeast gene *RAS2^{val19}*. This gene contains a mutation equivalent to that of mammalian *H-ras^{val12}*, one of the oncogenic forms of *H-ras*. Yeast with this mutation display several abnormal phenotypes, including failure to arrest in the G₁ phase of the cell cycle, failure to sporulate, sensitivity to starvation, and sensitivity to heat shock (2, 8). Because increased expression of adenosine 3',5'-monophosphate (cAMP) phosphodiesterase genes can suppress these phenotypes (9, 10), we expected that interfering forms of adenylyl cyclase might do likewise. We therefore randomly mutagenized a plasmid that expresses *CYR1* under the control of the strong promoter of the alcohol dehydrogenase gene *ADH-I* (11). The plasmid was passed through a mutator strain of *Escherichia coli* (12), and the resulting “library” of mutant plasmids was transformed into a *S. cerevisiae* strain, TK161-R2V, containing *RAS2^{val19}*. Transformants were scored for heat-shock sensitivity. In two screens of separate libraries of about 1000 transformants each, we isolated 10 and 13 plasmids that conferred heat-shock resistance on the *RAS2^{val19}* strain (13).

The ease with which interfering mutations could be isolated suggested that many different mutations in the adenylyl cyclase gene might give rise to interfering plasmids. The most obvious class of mutations that might be thought to be effective would be those lacking catalytic function, so we tested directly if such mutations would be interfering. The catalytic region of adenylyl cyclase is in the COOH-terminal 417 amino acids (14). We constructed a plasmid that had this region deleted and found it to be interfering (13). Further interfering constructs were

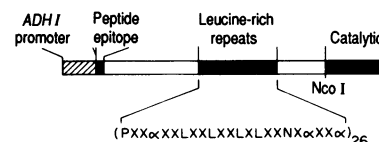
made by introducing a frameshift mutation at the *Nco I* restriction site located at the codon for amino acid 1608, just upstream of the catalytic region. We assayed for heat-shock sensitivity and found that *RAS2^{val19}* cells containing either a marker plasmid or a plasmid expressing large amounts of full-length adenylyl cyclase did not recover from heat shock, but cells containing a plasmid expressing the defective adenylyl cyclase with the frameshift mutation did recover (Fig. 1). Thus, the presence of an abundance of a catalytically inactive form of adenylyl cyclase appeared to inhibit the activity of the remaining wild-type protein.

To define the smallest region of adenylyl cyclase that possesses interfering activity, we

constructed a series of deletions that contained the frameshift mutation at the *Nco I* site (Fig. 2). The first series consisted of deletions from the 5' end of the gene. Molecules with deletions up to the codon for amino acid 605 retained full interfering activity. A molecule with a deletion up to amino acid codon 733 was partially active and those with deletions beyond codon 733 were not active (Fig. 2, group I). In the next series we began with the mutant gene deleted through the codon for amino acid 605 and made further deletions beginning from the *Nco I* site toward the 5' end. Molecules deleted from the *Nco I* site toward codon 1302, and thus encoding proteins consisting of amino acids 606 to 1301, were fully interfering (Fig. 2, group II). In the third series, deletions within the region between the codons for amino acids 606 and 1301 were made. Molecules with these mutations were not active (Fig. 2, group III).

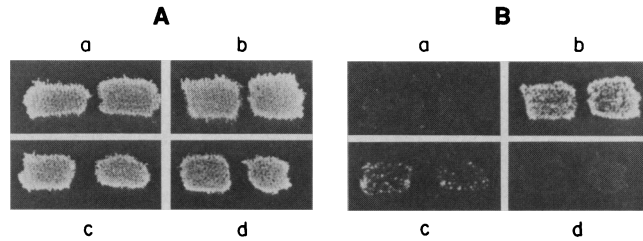
The region of adenylyl cyclase between amino acids 606 and 1301 contains 26 copies of a 23-amino acid repeating unit (from amino acid position 733 to 1301) that is rich in aliphatic (especially leucine) residues and is punctuated by one residue each of proline and asparagine (Fig. 2). Expression of this repeat region is the probable cause of interference. Deletion of amino acids 606 through 733 has little effect on interference, but deletions within the repeat

Fig. 2. Deletion analysis of interfering adenylyl cyclase. Deletions were made in the adenylyl cyclase gene starting with the plasmid pYCYR (27). The *Nco I* frameshift mutation was then introduced. This mutation destroyed catalytic function as assayed both by genetic complementation and biochemical analysis. Each frameshift construct was tested for interference as described in the legend to Fig. 1. The major structural features of the gene product are as follows: peptide epitope, a nine-amino acid monoclonal antibody-epitope fused to the NH₂-terminal sequence (24); leucine-rich repeats of the motif shown (P, Pro; L, Leu; N, Asn; α, Met, Ile, Leu, or Val; and X, any residue); catalytic region required for adenylyl cyclase activity as previously mapped (14). The scale used to assess interference is as follows: + + +, viability of heat shock-treated cells indistinguishable from wild-type cells; + +, partial interference; and -, no detectable interference.



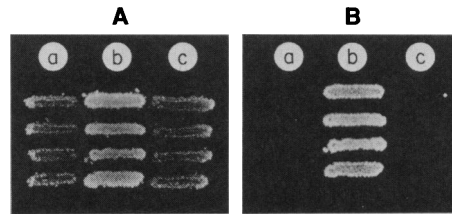
Plasmid	Codons deleted	Interference
pYCYRfs	None	+ + +
I		
pYC Δ10fs	41-605	+ + +
pYC Δ1fs	1-605	+ + +
pYC Δ2fs	1-733	+ +
pYC Δ3fs	1-1038	-
pYC Δ3ofs	26-1234	-
pYC Δ4fs	1-1301	-
II		
pYC Δ10fs	1-605, 1558-1608	+ + +
pYC Δ9fs	1-605, 1509-1608	+ + +
pYC Δ8fs	1-605, 1458-1608	+ + +
pYC Δ7fs	1-605, 1407-1608	+ + +
pYC Δ6fs	1-605, 1358-1608	+ + +
pYC Δ5fs	1-605, 1302-1608	+ + +
III		
pYC Δ11fs	1-605, 887-1065	-
pYC Δ13fs	1-605, 743-1065	-
pYC Δ12fs	1-605, 885-1233	-
pYC Δ14fs	1-605, 741-1233	-

Fig. 3. Competition between plasmids expressing $RAS2^{val19}$ and interfering adenylyl cyclase. The $RAS2^{val19}$ strain TK161-R2V was transformed with the indicated plasmids. Duplicate patches of transformants were tested for heat-shock sensitivity as described in the legend to Fig. 1.



1. Two plasmids were present in each transformant and were maintained by selection for growth on synthetic medium lacking leucine and histidine. The four plasmids used in the experiment were YEp13 (a yeast vector containing the auxotrophic marker *LEU2*), pHV1 (a yeast vector containing the auxotrophic marker *HIS3*) (7), pYCYRf (a yeast plasmid expressing interfering adenylyl cyclase and *LEU2*), and pRV1 (a yeast plasmid expressing $RAS2^{val19}$ and *HIS3*). The latter plasmid was constructed by inserting a 2.3-kb Hind III–Eco RV fragment containing the $RAS2^{val19}$ gene into the Sma I site of pHV1. (A) No heat shock; (B) heat shock at 55°C for 10 min. The plasmids in each strain were (a) YEp13 and pHV1, (b) pYCYRf and pHV1, (c) pYCYRf and pRV1, and (d) YEp13 and pRV1.

Fig. 4. Effect of an interfering adenylyl cyclase gene on heat-shock sensitivity in a yeast strain that lacks *RAS1* and *RAS2*. The indicated plasmids were introduced into strain DJ39-3D (*Mata leu2 ras1::TRP1 ras2::ADE8 pde1::URA3 pde2::HIS3*) or into the $RAS2^{val19}$ strain TK161-R2V. DJ39-3D is sensitive to heat shock as a result of the disruption of the cAMP phosphodiesterase genes (10). Four patches on synthetic medium lacking leucine were made from independent transformants. The transformants were tested for heat-shock sensitivity as described in Fig. 1. The plate in (A) was not subjected to heat shock; the plate in (B) was subjected to a 10-min heat shock. The strains and transforming plasmids were as follows: (a) DJ39-3D transformed with YEpM4 (a yeast vector plasmid expressing the *LEU2* auxotrophic marker) (10); (b) TK161-R2V transformed with YEpPDE2 (a *LEU2* plasmid expressing the yeast phosphodiesterase gene *PDE2*) (9); and (c) DJ39-3D transformed with pYCYRf (a plasmid expressing interfering adenylyl cyclase).



abolish interference. This repeated consensus sequence is also found in tandem repeats present in several other proteins. These include the *Chaoptin* (15) and *Toll* (16) gene products of *Drosophila melanogaster*, the porcine ribonuclease inhibitor (17), the α (18) and β (19) chains of the human serum glycoprotein Ib, the human serum α_2 -glycoprotein (20), and the lutropin-choriogonadotropin receptor (21). All these proteins are thought to bind other proteins. The glycoprotein Ib α chain (also known as the platelet receptor for the von Willebrand factor) and the lutropin-choriogonadotropin receptor are both thought to require the leucine-rich repeats for protein binding (18, 21). The repeat region of adenylyl cyclase does not form a “leucine zipper” according to the model of Landschulz *et al.* (22) because the leucines are not present at every seventh position on an α helix. The leucine-rich repeats may, however, form an analogous structure. Trans-dominant mutations in leucine-zipper proteins have also been reported. These mutations result in partially functional proteins capable of interfering with their appropriate targets (23).

We have postulated that interference by the truncated adenylyl cyclase occurs by sequestering a functioning component of the signal transduction pathway. To test if this component is the RAS protein itself, we

investigated if overexpression of $RAS2^{val19}$ could overcome the interference of defective adenylyl cyclase. We examined four pairwise combinations of plasmids transformed into a $RAS2^{val19}$ strain: these plasmids included one expressing $RAS2^{val19}$, one expressing an interfering cyclase, and two control plasmids carrying the appropriate auxotrophic markers. Transformed strains were tested for heat-shock sensitivity. The two control plasmids together had no effect on heat-shock sensitivity, whereas the interfering adenylyl cyclase fully suppressed heat-shock sensitivity. The $RAS2^{val19}$ plasmid had no effect on heat shock–sensitivity. However, the $RAS2^{val19}$ plasmid largely overcame the interference caused by the truncated adenylyl cyclase (Fig. 3). Cells that contained the interfering adenylyl cyclase and overexpressed the $RAS2^{val19}$ protein were largely heat shock–sensitive.

This last result suggested that interfering adenylyl cyclase acts by sequestering $RAS2^{val19}$ protein. To test this further, we determined if active RAS protein was required for interference. Yeast strains that lack both *RAS1* and *RAS2* and also both genes encoding the cAMP phosphodiesterases, *PDE1* and *PDE2*, are heat shock–sensitive (10). This phenotype presumably reflects the large amounts of intracellular cAMP, which is produced by basal levels of

adenylyl cyclase activity in the absence of RAS proteins and accumulates because of the absence of cAMP hydrolysis. The heat-shock sensitivity of a *ras1 ras2 pde1 pde2* strain was not affected by the interfering adenylyl cyclase plasmid (Fig. 4). As a positive control in this experiment, a plasmid expressing *PDE2* was shown to suppress the heat shock-sensitivity in the $RAS2^{val19}$ strain TK161-R2V. Thus, interference was not observed in the absence of RAS proteins.

Our results suggest that expression of the repeat region of adenylyl cyclase competes for RAS function. The simplest hypothesis consistent with our data is that this region of adenylyl cyclase forms a complex that sequesters RAS. There are several ways to envision this occurring. (i) RAS proteins might interact directly with the repeat region. (ii) Functional adenylyl cyclase might form an ineffective complex with the repeat region. (iii) Other proteins might combine with the repeat region to form a complex capable of binding RAS proteins. We have found that other proteins do complex with yeast adenylyl cyclase (24).

We have demonstrated that, in yeast, a defective protein target for RAS action can be used to block the RAS signaling pathway. This observation may serve as the basis of a genetic assay for the identification of other targets for RAS action. Therefore, we have searched libraries expressing mammalian cDNAs in yeast for genes suppressing heat-shock sensitivity (25).

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 27. pYCYR was constructed by first cloning the 4-kb Hind III fragment of pEF-CYR1 (24), encoding the COOH-terminal region of adenyl cyclase, into the Hind III restriction site of YEpl3 to yield the plasmid pYC. The 4.5-kb Bam HI-Xho I fragment of pEF-CYR1, containing the *ADH-I* promoter and region encoding the NH₂-terminal region of adenyl cyclase, was then cloned into pYC, which had been digested with Bam HI and Xho I, to create

pYCYR. pYCYR differs from pEF-CYR1 only in the vector used to carry adenyl cyclase. pEF-CYR1 carries the *TRP1* auxotrophic marker, whereas pYCYR carries *LEU2*. Both plasmids use the yeast *ADH-I* promoter to express an adenyl cyclase fusion protein. This protein has a small peptide epitope fused to the NH₂-terminus of adenyl cyclase. The epitope is recognized by monoclonal antibody 12CA5, which can be used to monitor and purify the fusion protein (24). The codons deleted are indicated by means of the numbering system of Kataoka *et al.* (14). In some cases two regions were deleted. Precise deletions were made by reconstructing the parent plasmid with combinations of restriction endonuclease fragments and the desired DNA products of polymerase chain reactions [R. K. Saiki *et al.*, *Science* **239**, 487 (1988)]. Details of the deletions are described elsewhere (J. Colicelli, J. Field, R. Ballister, N. Chester, M. Wigler, in preparation). Each construct was first tested for function by complementation in adenyl cyclase-deficient or RAS-deficient strains. Constructs were tested by immunoprecipitation of adenyl cyclase activity

with monoclonal antibody 12CA5. In some cases the enzyme was additionally tested by protein immunoblots or by affinity purification, in each case with the use of monoclonal antibody. After it was established that the deleted plasmids produced a catalytically functional adenyl cyclase molecule, a frameshift mutation was introduced at the Nco I restriction site. This was performed by digestion with Nco I, filling in the ends with Klenow fragment, followed by ligation. This procedure was monitored by the creation of an Nsi I site formed by the ligation of the filled-in ends of the Nco I site.

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Two Distinct Transcription Factors That Bind the Immunoglobulin Enhancer μ E5/ κ E2 Motif

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Activity of the immunoglobulin heavy and κ light chain gene enhancers depends on a complex interplay of ubiquitous and developmentally regulated proteins. Two complementary DNAs were isolated that encode proteins, denoted ITF-1 and ITF-2, that are expressed in a variety of cell types and bind the μ E5/ κ E2 motif found in both heavy and κ light chain enhancers. The complementary DNAs are the products of distinct genes, yet both ITF-1 and ITF-2 are structurally and functionally similar. The two proteins interact with one another through their putative helix-loop-helix motifs and each possesses a distinct domain that dictates transcription activation.

THE IMMUNOGLOBULIN HEAVY chain (IgH) enhancer activates transcription of rearranged heavy chain genes (1). In transfection experiments it stimulates transcription from a variety of promoters, but only in cells of the lymphoid lineage (primarily B cells). This activity is mediated through several protein binding sites. Four of these sites, μ E1, μ E2, μ E3, and μ E4, were defined initially by *in vivo* footprinting (2). Two others, octa (3) and μ EBP-E (4), were first defined *in vitro*. Deletions or mutations of these sites generally reduce overall enhancer activity (5-7). However, mutation analyses also suggest that the sequence motifs identified by DNA binding assays do not account for all of the IgH enhancer activity (5, 6). In particular, a deletion that destroys both the μ E1 and μ E2 sites and removes the 24 bp between these two sites has a more deleterious effect in B cells than clustered point mutations that simultaneously destroy these two motifs (6). This deletion results in an increase in en-

hancer activity in mouse L cells, suggesting that the region between μ E1 and μ E2 may comprise a negative regulatory element as well (6, 8). An examination of the nucleotide sequence between μ E1 and μ E2 reveals

an additional E-related motif, referred to as μ E5 (9). Although this site closely resembles the κ light chain enhancer κ E2 site, protein binding to the μ E5 site has not been detected with crude nuclear extracts (5, 10).

To study the function of this region of the enhancer in more detail, we have isolated cDNAs that encode its cognate DNA binding proteins. We used an oligonucleotide bearing both the μ E5 and μ E2 sites to screen a B cell-derived λ gt11 cDNA library (11). We analyzed in detail two phage isolates, denoted E2-2 and E2-5, that had binding activity for the oligonucleotide. A series of experiments employing mutant oligonucleotides confirmed that the DNA binding activity encoded by each phage was specific for the μ E5 motif within the oligonucleotide, as well as for a κ E2 motif carried on a different oligonucleotide (12).

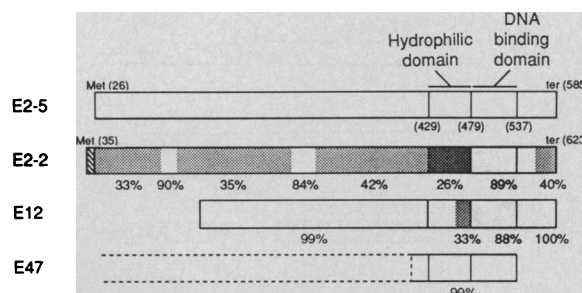


Fig. 1. Comparison of proteins that bind the μ E5/ κ E2 site. A schematic comparison of the amino acid sequences deduced from the E2-5 cDNA, the E2-2 cDNA (GenBank accession numbers M30314 and M30313, respectively), and two related cDNAs (E12 and E47) isolated by Murre *et al.* (9) is shown. Amino acid positions are shown in parentheses, with the terminal Eco RI sites of the E2-5 and E2-2 cDNAs representing amino acids

one and two. Amino acid similarity of regions within each protein, relative to the protein encoded by E2-5, is indicated by percent identity and by relative shading, the lighter the shading, the more related (gaps were introduced to optimize the alignments). The dashed lines of clone E47 indicate the region of that cDNA not sequenced. A region of high charge density (hydrophilic domain) and the DNA binding domain as determined by Murre *et al.* (9) are shown. Phage clones E2-2 and E2-5 were isolated from a human B cell derived λ gt11 cDNA library (Clontech, Palo Alto, California) as described (11), with the following modification. The probe used for screening, the μ E5 + μ E2 oligonucleotide (5'-AG-AACACCTGCAGCAGCTGGCAGG-3'; μ E5 and μ E2 sites underlined), was end-labeled with [γ -³²P]-adenosine triphosphate and then ligated to form random concatamers. Nucleotide sequences (both strands) were determined using the dideoxy method and Sequenase T7 DNA polymerase (USB, Cleveland, Ohio) on single-stranded M13 subclones generated with various restriction enzyme fragments. Specific oligonucleotides were also synthesized to use as sequencing primers. Nucleotide sequences are available upon request.

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