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33. We thank H. L. Moses, B. L. M. Hogan, L. Sealy, and W. J. Pledger for critical reading of the manuscript, A. Meymandi and N. E. Olashaw for helpful suggestions, L. Anderson for photographic assistance, and E. Fields, L. Bruno, D. Miller, and C. Robinson for technical assistance. We thank W. J. Pledger for his gift of PDGF, and M. Magnuson for pPLFCAT. The antiserum directed against the M-peptide of Fos was a generous gift from T. Curran, Roche, Nutley, NJ. L.D.K. is supported by a National Cancer Institute institutional training grant and J.T.H. is a PEW Scholar supported by the PEW Memorial Trust. L.M.M. is supported by an American Chemical Society Junior Faculty Research Award and by NIH.

16 August 1988; accepted 18 October 1988

Mitochondrial DNA Mutation Associated with Leber's Hereditary Optic Neuropathy

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Leber's hereditary optic neuropathy is a maternally inherited disease resulting in optic nerve degeneration and cardiac dysrhythmia. A mitochondrial DNA replacement mutation was identified that correlated with this disease in multiple families. This mutation converted a highly conserved arginine to a histidine at codon 340 in the NADH dehydrogenase subunit 4 gene and eliminated an Sfa NI site, thus providing a simple diagnostic test. This finding demonstrated that a nucleotide change in a mitochondrial DNA energy production gene can result in a neurological disease.

LEBER'S HEREDITARY OPTIC NEUropathy (LHON) is a maternally inherited form of central optic nerve death associated with acute bilateral blindness. Cardiac dysrhythmia is also common and peripapillary microangiopathy is frequently seen in presymptomatic individuals. The age of onset ranges from adolescence to late adulthood; median age of onset is 20 to 24 years (*1–3*).

In numerous large pedigrees, LHON patients have been found to be related exclusively through the maternal lineage (*4*). No case of paternal transmission has been demonstrated. However, in most pedigrees, expression is variable and there is a bias toward males exhibiting ophthalmological problems (*1–5*).

The human mitochondrial DNA (mtDNA) is also maternally inherited (*6*). It codes for a large and small rRNA, 22 tRNAs, and 13 polypeptides of oxidative phosphorylation; ND1 to ND6 of complex I, cytb of complex III, COI to COIII of complex IV, and adenosine triphosphatase (ATPase) subunit 6 and subunit 8 of complex V (*1, 7–9*). Further, chronic exposure of primates and rodents to low levels of respiratory inhibitors results in optic neuropathy and basal gangliar lesions (*1, 10*). Thus, both the symptoms and inheritance of LHON suggest that this disease is the product of an mtDNA mutation.

Restriction analysis of lymphoid and muscle mtDNA samples ruled out deletions (*11*) as the cause of LHON. Therefore, candidate mutations for LHON were sought by cloning and sequencing the mtDNA from the blind proband of a large, black LHON pedigree from Georgia (Fig. 1). Since most reported LHON pedigrees are Caucasoid, analysis of a black LHON mtDNA minimized the chances of associating the disease with a rare ethnic-specific sequence polymorphism. Eighty-five percent of the LHON mtDNA coding region was sequenced. Comparison with the "normal" Cambridge mtDNA sequence (*7*) revealed 25 base substitutions, eight of which altered amino acids (Table 1).

A replacement mutation in the LHON mtDNA sequence would be implicated in the disease if it (i) changed a highly evolutionarily conserved amino acid (Table 1), (ii) was frequently found in LHON patients, and (iii) and was not found in normal

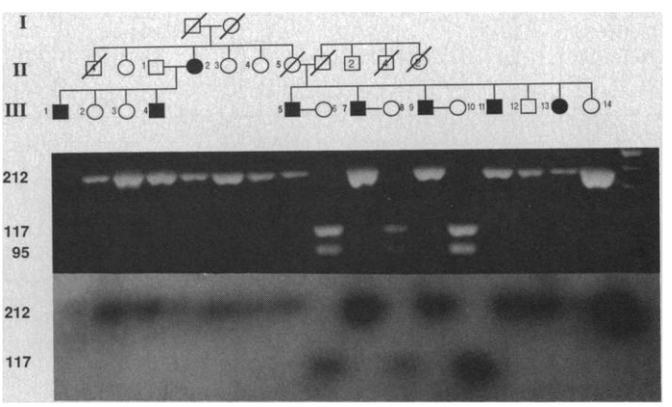

Fig. 1. Black LHON pedigree from the Georgia family. Filled symbols indicate central vision loss, open indicate no reported vision loss. Roman numerals indicate generations. Numbers in symbols indicate multiple siblings. Numbers outside symbols indicate family members studied. The mtDNA of proband III5 was cloned and sequenced. The middle panel shows the Sfa NI digestion patterns of a 212-bp PCR product that includes the nt11778 mutation. DNA for amplification was prepared from fractionated blood platelets (*29*), from white blood cells, or from transformed lymphoblasts. The samples were enriched for white blood cell and lymphoblast mtDNAs by Hirt extraction (*30*). The PCR reaction included 1 ng of target DNA and 2.5 units of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) (*20*). Approximately 200 ng of DNA were digested overnight at 37°C with 1 unit of Sfa NI. Digestion products were separated on 2.5% NuSieve plus 0.9% SeaKem agarose (FMC, Rockland, ME) gels. The lower panel shows the DNA blot hybridization of the Sfa NI digestion products with a 5′ end-labeled internal oligonucleotide. Sizes are expressed in base pairs.

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individuals from the general population (Table 2). Six of the mutations [nucleotide positions (nt): 8701, 9559, 10398, 13702, 14199, and 12385] did not fulfill these criteria and thus were unlikely to be causally related to LHON. The amino acids affected by substitutions at nt8701, nt10398, nt14199, and nt12385 are not conserved between species (*12–15*), whereas those at nt9559 and nt13702 are the same between LHON and other mammals, implying errors in the Cambridge sequence (Table 1). Furthermore, the LHON substitution at nt9559 was found in 11 other human mtDNA sequences (*16*), that at nt10398 was found in HeLa mtDNA (*17*), and those at nt10398, nt13702, and nt14199 were identified in normal mtDNAs by the restriction enzymes Dde I (59 of 133) (*18*), Hae III (4 of 134) (*18*), and Hinc II (116 of 116) (*19*), respectively. The lack of correlation between LHON and mutations nt13702 and nt14199 was confirmed by restriction digestion of polymerase chain reaction (PCR)–amplified (*20*) mtDNAs from a panel of ten controls and six patients. This analysis was extended to nt8701 and nt12385 by hybridization with pairs of oligonucleotides (Table 2).

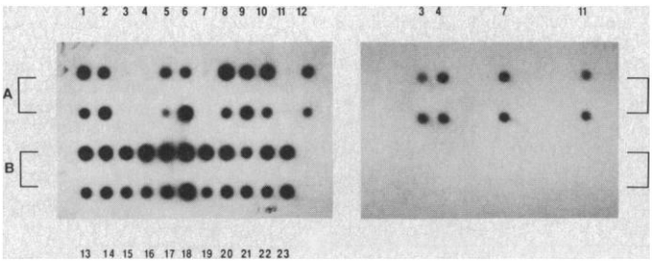

Fig. 2. Analysis of the nt9163 mutation by oligonucleotide hybridization to a 424-bp PCR fragment. Duplicate spots (pairs A or B) were made with 10 to 20 ng of each PCR fragment. Left panel shows Cambridge probe and right shows LHON probe (Table 2). Control individuals are samples 1, 2, 5, 6, 9, 10, 15, 18, 19, and 21. LHON individuals are 3, 4, 7, 8, 11, 12, 13, 14, 16, 17, and 20. LHON plus IBSN individuals are 22 and 23.

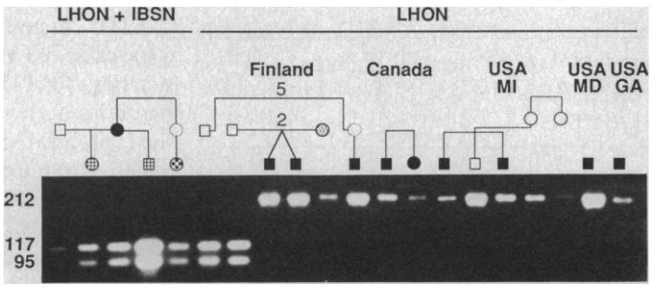

Fig. 3. Sfa NI analysis of the nt11778 mutation from LHON pedigrees of North America and Europe (pedigrees 2 and 5 of Finland). Filled symbols, bilateral blindness; curved lines, microangiopathy; open checks, dystonia associated with IBSN; and filled checks, bilateral blindness and dystonia. Sizes are expressed in base pairs.

Table 1. Eight LHON replacement substitutions are compared to the Cambridge (Camb) sequence (*7*), partial human sequences encompassing nt9559 (*16*) and nt10398 (*17*), primate (*14*), bovine (*12*), mouse (*13*), and rat (*15*) sequences. Additional nucleotide substitutions, methods, and abbreviations are in (*26*). AA, amino acid.

Sequences	LHON replacement mutations					
	ATPase 6		ATPase 6		COIII	
	nt	AA	nt	AA	nt	AA
LHON	8701(G)	A	9163(A)	I	9559(C)	P
Human, Camb	8701(A)	T	9163(G)	V	9559(G)	R
Human, other (*15*)			9559(C)	P		
Bovine	8464(A)	S	8926(G)	V	9322(C)	P
Mouse	8101(C	L	8563(G)	V	8959(C)	P
Rat	8976(T)	L	9438(G)	V	9834(C)	P
	ND3		ND5		ND6	
	nt	AA	nt	AA	nt	AA
LHON	10398(G)	A	13702(C)	R	14199(T)	T
Human, Camb	10398(A)	T	13702(G)	G	14199(G)	P
Human, other (*16*)	10398(G)	A				
Bovine	10162(A)	T	13474(C)	R	13963(A)	S
Mouse	9795(A)	T	13101(C)	R	13593(A)	S
Rat	10673(A)	T	13984(C)	R	14473(A)	S
	ND4		ND5			
	nt	AA	nt	AA		
LHON	11778(A)	H	12385(T)	S		
Human, Camb	11778(G)	R	12385(C)	P		
Chimpanzee	99(G)	R	705(C)	L		
Gorilla	99(G)	R	705(C)	P		
Orangutan	99(G)	R	705(C)	P		
Gibbon	99(G)	R	705(C)	P		
Bovine	11547(G)	R	12157(A)	M		
Mouse	11179(G)	R	11784(T)	S		
Rat	12061(G)	R	12667(A)	T		

The substitutions at nt9163 and nt11778, by contrast, could not be eliminated upon consideration of available sequence or restriction site polymorphism data. In LHON, the nt9163 mutation in ATPase 6 converts the valine, 14 amino acids from the 3′ end of the polypeptide, to an isoleucine. This valine is conserved in all mammalian mtDNAs (Table 1). To determine if this was the LHON mutation, a pair of oligonucleotide probes was hybridized to an ATPase 6 PCR fragment amplified from 11 controls and 12 LHON patients from six pedigrees (Fig. 2 and Table 2). The LHON probe only hybridized to mtDNAs from members of the Georgia pedigree. The remaining patients and controls hybridized to the Cambridge probe. Hence, this mutation did not correlate with LHON.

The mutation at nt11778 in ND4 converts a G in the Cambridge sequence to an A in the LHON mtDNA. This changes the 340th amino acid from an arginine to a histidine. This arginine is highly conserved between species (Table 1). The mutation also removes an Sfa NI site and thus can be readily identified by Sfa NI digestion of a 212-bp PCR fragment encompassing the mutation. The PCR fragments of the Georgia LHON maternal lineage were not cut with Sfa NI, a result consistent with the sequence data. However, the amplified mtDNAs from the spouses were cut into the expected 117- and 95-bp fragments (Fig. 1). The origin of the 212- and 117-bp fragments was verified by hybridization with an oligonucleotide probe homologous to an internal sequence (Fig. 1). Amplified mtDNAs from ten controls and six LHON patients revealed that all controls retained the Sfa NI site, whereas all LHON patients lacked the site (Table 2). Hence, the nt11778 mutation did correlate with

LHON.

To determine the generality of this relation, a total of 45 independent controls (10 blacks, 10 Asians, 14 Caucasians, and 11 Finns) were tested. Every control sample retained the Sfa NI site (*21*).

These control results were compared to those of 11 independent LHON lineages. The LHON pedigrees were collected from diverse geographic and ethnic backgrounds so that it would be unlikely that they would all by chance harbor the same rare ethnic mtDNA restriction fragment length polymorphism. Nine of the 11 LHON pedigrees lacked the Sfa NI site. Four of these were from different regions of North America. From the Georgia pedigree, we tested 13 maternal relatives, 7 of whom were blind. All lacked the Sfa NI site (Fig. 1). From a Michigan pedigree, we tested five maternal relatives; two were blind and all lacked the Sfa NI site. From a Maryland pedigree, we examined the blind proband, and he lacked the site. Finally, from a Canadian pedigree, we tested a blind brother and sister, and both lacked the site. The five remaining LHON pedigrees that lacked the site were from Finland (*21*). We tested one blind member from each of four Finnish pedigrees (pedigrees 1, 3, 4, and 5), and all four lacked the site. We examined eight maternal relatives, seven blind and one with microangiopathy, in an additional pedigree (Finnish pedigree 2). All of these individuals lacked the site. Examples of these results are shown in Fig. 3. Overall, from these nine pedigrees we have tested 33 individuals (23 blind) and confirmed that all had the LHON mutation at nt11778.

Two of the 11 LHON pedigrees were discordant, however. The first of these was from the Human Genetic Mutant Cell Repository (Camden, New Jersey). We tested the cell line GM3857 derived from one of two blind brothers, and it retained the Sfa NI site. The second was a large pedigree from Finland with multiple affected individuals (pedigree 6). We tested two blind cousins and both retained the site. Even with these discordant cases, however, the association between LHON and the nt11778 mutation was highly significant ($P \leq 0.001$ by χ^2 test).

Five points support the hypothesis that the mtDNA mutation at nt11778 is the cause of LHON. First, both LHON and the mtDNA are exclusively maternally inherited. Second, the nt11778 mutation is found in multiple independent LHON lineages, but not in numerous unaffected control lineages. Third, the nt11778 mutation changes a highly conserved amino acid in a region of the genome where there is a greater than 85% amino acid homology between mouse, cow, and human (*7, 12, 13*). Fourth, all other observed LHON mtDNA substitutions were ruled out. Fifth, the mutation would alter, but probably not eliminate, the respiratory function of ND4. This dysfunction could mimic the effects of chronic respiratory inhibitor treatment. Therefore, it seems likely that the nt11778 mutation is the cause of LHON in most families.

Although the nt11778 mutation does result in LHON, the existence of two discordant pedigrees implies that it may not be the only cause. This implication was confirmed by analyzing four individuals from a LHON plus infantile bilateral striatal necrosis (IBSN) pedigree in which optic neuropathy without presymptomatic microangiopathy is associated with dystonic rigidity and basal gangliar lesions (*22*). All four LHON plus IBSN individuals retained the Sfa NI site (Fig. 3), confirming that this disease is different from LHON. The two discordant "LHON" pedigrees might then be LHON plus IBSN pedigrees that have not yet expressed the IBSN phenotype, or they may be the product of an alternative mutation that can result in LHON.

Although there is an excellent correlation between LHON and the loss of the Sfa NI site between maternal lineages, there is a much less clear association between the loss of the Sfa NI site and the optic atrophy and cardiac dysrhythmia observed within pedigrees (Figs. 1 and 3). Since the nt11778 mutation was found to be homoplasmic in all LHON pedigrees examined, the variable symptoms cannot be the result of replicative segregation of a mixed mutant and wild-type mtDNA population, a phenomenon that best accounts for the variable phenotypes observed in our myoclonic epilepsy and ragged red fiber disease pedigree (*23*). Therefore, the nt11778 mutation appears to be necessary but not sufficient for overt symptoms. This conclusion implies that additional factors are involved in the expression of the mutant phenotype. The sex bias in symptoms could result from a sex-related physiological difference, an X-chromosome dosage effect, or a recessive X-linked modulating gene. Furthermore, environmental stresses that reduce respiratory capacity, for example, smoking (*24*), might augment the expression of the mutation. If this is the case, then it is possible that metabolic therapies that increase cellular respiratory metabolism (*25*) might reduce the risk of deleteri-

Table 2. Preliminary screen of mutations in ten controls and six LHON pedigrees. A (+) indicates the published sequence (*7*) and a (−) the LHON sequence. For mutations with informative restriction enzyme sites (nt 13702, 14199, and 11778), the (+) or (−) is also indicative of the presence or absence of the site. NT indicates not tested. Mutations at nt 13702, 14199, and 11778 were screened by restriction endonuclease digestion. Ten units of Hae III (nt13702) and 5 units of Hinc II (nt14199) were used for 16 hours at 37°C. Sfa NI digestion (nt11778) and electrophoresis parameters are in Fig. 1 legend. The mutations at nt 8701, 9163, and 12385 were screened by differential oligonucleotide hybridization. For nt8701, the Cambridge probe was CAAATGATAACCATACACA and the LHON probe was TGTGTATGGCTATCATTTG; for nt9163, they were GTGTGAAAACGTAGGCTTG and CAAGCCTACATTTTCACAC; and for nt12385, they were TCCCTAATTCCCCCCATCC and GGATGGGGGAAATTAGGGA. Filters were prepared by the alkaline dot blot method (*27*). All filters were prehybridized and hybridized at 44°C except for the Cambridge probe at nt8701, which was hybridized at 35°C. Wash temperatures (*28*) were 47°C (nt8701 Cambridge), 52°C (nt8701 LHON), 56°C (nt9163 Cambridge), 54°C (nt9163 LHON), 56°C (nt12385 Cambridge), or 55°C (nt12385 LHON).

Samples	Informative nucleotide positions*					
	8701 (374)	9163 (424)	13702 (786)	14199 (692)	11778 (212)	12385 (598)
Controls						
African 1	−	+	−	−	+	+
African 2	−	+	−	−	+	+
HeLa	−	+	−	−	+	−
U.S. black	−	+	−	−	+	−
Chinese 1	−	+	−	−	+	−
Chinese 2	−	+	NT	−	+	+
European 1	+	+	−	−	+	−
European 2	+	+	−	−	+	−
European 3	+	+	−	−	+	−
Finnish	+	+	−	−	+	−
LHON						
Finnish Ped. 1	+	+	NT	−	−	+
Finnish Ped. 2	+	+	−	−	−	+
Finnish Ped. 3	+	+	−	−	−	+
GA Ped. (III9)	−	−	−	−	−	−
GA Ped. (III5)	−	−	−	−	−	−
MD Ped. 1	+	+	−	−	−	−

*The sizes (in base pairs) of the PCR fragments used are shown in parentheses.

ous symptoms in as yet unaffected family members.

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21. Control samples were from unrelated maternal lineages. These included 10 blacks (2 Africans, 1 HeLa cell line, and 7 American blacks), 10 Asians (9 Chinese and 1 Japanese), 14 Caucasians (Europeans), and 11 Finns (paternal lineage controls from Finnish pedigrees 1 to 4). Finnish pedigrees 1, 2, and 6 of this study are pedigrees A, D, and B, respectively, in (*3*). Patients from the five Finnish pedigrees are individual VI11 of pedigree 1; individuals V5, V7, V8, V12, V18, VI6, VI7, and V3 of pedigree 2 (Fig. 3); and the probands of pedigrees 3, 4, and 5. We tested individuals IV4 and IV17 from the discordant Finnish pedigree 6.
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26. Additional base substitutions (Cambridge to LHON) included two structural (G to A at nt1438, in the 12*S* rRNA, and G to A at nt2706, in the 16*S* rRNA) and 15 synonymous mutations: G to T, nt3423; A to G, nt4169; T to C, nt6221; C to T, nt6587; C to T, nt7028; T to C, nt9540; T to C, nt10370; A to G, nt10819; T to C, nt10873; T to C, nt11335; C to T, nt12705; A to T, nt13893; T to C, nt14152; T to C, nt14212; and A to C, nt15256. Peripheral lymphocytes from III5 (Fig. 1) were isolated by Ficoll-Hypaque gradients and transformed with Epstein-Barr virus (*8*). Mitochondria were isolated by differential centrifugation, and the mtDNA was purified by two sequential CsCl–ethidium bromide gradients [R. E. Giles, I. Stroynowski, D. C. Wallace, *Somatic Cell Genet.* **6**, 543 (1980)]. Purified mtDNA was digested with Taq I or various combinations of Bam HI, Eco RI, Hind III, Pst I, or Xba I, and the fragments were cloned into M13. MtDNA fragments were identified by hybridization with regional probes [(*17*); J. E. Garrison, E. Hardeman, R. Wade, L. Kedes, P. Gunning, *Gene* **38**, 177 (1985)] and partial sequencing. All sequencing was done by the dideoxy chain termination procedure [F. Sanger, A. R. Coulson, B. G. Barrell, A. J. H. Smith, B. A. Roe, *J. Mol. Biol.* **143**, 161 (1980)]. Sequences read and compiled [C. Queen and L. J. Korn, *Nucleic Acids Res.* **12**, 581 (1984)] were nt 1220–3899, 3945–5269, 5275–8005, 8284–13610, 13625–14260, and 14803–15260. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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31. We thank R. P. Erickson (Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI), L. I. Grossman (Department of Molecular Biology and Human Genetics, Wayne State University School of Medicine, Detroit, MI), H. A. Gardner (Oshawa General Hospital, Oshawa, Ontario), H. H. Kazazian, Jr. (Department of Pediatrics, Johns Hopkins University Hospital, Baltimore, MD), E. J. Novotny, Jr. (Department of Neurology, Yale University Medical School, New Haven, CT), and R. Spector (Atlanta, GA) for contributing LHON pedigree samples to this study. We also acknowledge the technical assistance of T. Horton. Supported by a grant from the Sigrid Juselius Foundation (E.N.), and an NIH grant (NS21328) and an Emory University Pediatric Research Foundation grant (D.C.W.).

5 July 1988; accepted 22 September 1988

Cyclic AMP–Responsive DNA-Binding Protein: Structure Based on a Cloned Placental cDNA

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Cyclic AMP (cAMP) is an intracellular second messenger that activates transcription of many cellular genes. A palindromic consensus DNA sequence, TGACGTCA, functions as a cAMP-responsive transcriptional enhancer (CRE). The CRE binds a cellular protein of 38 kD in placental JEG-3 cells. A placental λgt11 library was screened for expression of specific CRE-binding proteins with the CRE sequence as a radioactive probe. A cDNA encoding a protein of 326 amino acids with the binding properties of a specific CRE-binding protein (CREB) was isolated. The protein contains a COOH-terminal basic region adjacent to a sequence similar to the "leucine zipper" sequence believed to be involved in DNA binding and in protein-protein contacts in several other DNA-associated transcriptional proteins including the products of the c-*myc*, c-*fos*, and c-*jun* oncogenes and GCN4. The CREB protein also contains an NH_2-terminal acidic region proposed to be a potential transcriptional activation domain. The putative DNA-binding domain of CREB is structurally similar to the corresponding domains in the phorbol ester–responsive c-*jun* protein and the yeast transcription factor GCN4.

ACTIVATION OF SECOND MESSENGER pathways by hormones results in the transcriptional stimulation of many cellular genes (*1*). Phorbol esters (for example, 12-O-tetradecanoylphorbol 14-acetate, TPA) and cAMP activate the protein kinase C- and A-signaling pathways, respectively. Similar consensus DNA regulatory elements are involved in the stimulation of gene transcription by these agents (*2, 3*); the octameric cAMP-response element (CRE, 5′-TGACGTCA-3′) differs from the heptameric TPA-response element (TRE, 5′-TGAGTCA-3′) by only a single base. Earlier studies suggested that transcriptional stimulation by both cAMP and TPA can be mediated through a common DNA sequence present in the 5′ regulatory region of the enkephalin gene, 5′-TGCGTCA-3′ (*3*). However, a DNA-binding protein of 47 kD (AP-1 or c-Jun) was isolated and shown to mediate TPA but not cAMP induction of SV40 gene transcription through a mechanism involving sequence-specific binding to the TRE motif (*4*). Similarly, a 43-kD protein (CREB) was identified that binds to a CRE sequence in the 5′ regulatory region of the rat somatostatin gene (*5*). Utilizing in vitro mutagenesis, we have recently demonstrated that the cAMP- and phorbol ester–activated transcriptional responsiveness of these related DNA elements can be dissociated by the addition or subtraction of a single base, and further, that different DNA-binding proteins interact with these related

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