Cloning and Characterization of cDNAs Encoding Chicken Mitogen-Activated Protein Kinase Kinase Type 2, MEK2: Downregulation of MEK2 in Response to Inhibition of Mitochondrial DNA Expression†

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Received August 6, 1997; Revised Manuscript Received October 1, 1997⊗

ABSTRACT: The present work was initiated with the aim of identifying nuclear genes whose expression is sensitive to the mitochondrial DNA (mtDNA) status of transformed chicken DU24 cells. We cloned and sequenced cDNAs for the mitogen-activated protein kinase kinase type 2, MEK2, a protein involved in the mitogenic growth factor signal transduction pathway in vertebrates. Sequence comparisons between the chicken protein and its mammalian counterparts indicated that MEK2 proteins are highly conserved among vertebrates. Southern blot analysis of endonuclease-digested genomic DNA from primary chick embryo fibroblasts (CEF) suggested that *MEK2* is a single-copy gene in this vertebrate species. The steady-state level of *MEK2* transcripts is decreased in DUS3 mtDNA-less (*rho*^{0}) cells developed by long-term exposure of DU24 *rho*$^+$ cells to ethidium bromide (EtdBr). Run-on *in vitro* transcription assays and mRNA stability studies indicated that the decrease in *MEK2* mRNA content is associated with post-transcriptional regulation. In parental DU24 cells, *MEK2* mRNA content decreased after inhibition of mtDNA transcription by EtdBr and inhibition of translation on mitoribosomes by chloramphenicol (CAM). Cytoplasmic hybrids (cybrids) constructed by fusion of chicken *rho*^{0} cells with enucleated parental cells and CEF recovered a basal level of *MEK2* expression. The MEK2 protein content is decreased in DUS3 *rho*^{0} cells and in parental DU24 *rho*$^+$ cells treated with EtdBr and CAM for 6 days, while that of MEK1, a closely related kinase, remained unchanged. On the basis of these observations, we propose that mitochondria participate in the mitogenic signal transduction pathway in chicken cells through regulation of MEK2 expression.

There is growing evidence for the involvement of the mitochondrial genome (mtDNA[1]) to the cell phenotype of eukaryotes. Disorders of mtDNA that impair the respiratory capacity of human cells have been associated recently with a large number of clinically distinct pathologies, including maternally inherited and age-related diseases [for recent reviews, see Johns (1995) and Wallace (1994)]. In yeast, respiratory-incompetent cells induced by mutation in (*rho*$^-$) or depletion of (*rho*^{0}) mtDNA have also been shown to exhibit pleiotropic effects, including cell surface changes [for a review, see Wilkie et al. (1983)], analogous alterations being of importance in vertebrate tumorigenicity (Wilkie & Evans, 1982). We have shown that mtDNA is required to maintain the tumorigenic capacity of transformed avian cells (Zinkewich-Péotti et al., 1990, 1991). Collectively, these and other observations (Herzberg et al., 1993; Spelbrink et al., 1994) indicate that a number of phenotypic traits exhibited by unicellular eukaryotes and vertebrate cells accompany alterations of the mitochondrial genotype. The molecular mechanisms underlying the phenotypic response of eukaryotic cells to inhibition of mtDNA expression are presently unknown.

Recent research on nucleo−mitochondrial interactions in eukaryotes has focused on the expression of nuclear genes in cells depleted of or containing grossly altered mtDNA molecules. Upregulation of a variety of nuclear genes, some of them coding for proteins not related directly to mitochondrial structure and functions, has been identified in yeast *rho*$^-$ cells and in yeast and mammalian *rho*^{0} cells (Parikh et al., 1989; Wang & Brandiss, 1987; Parteladis & Mason, 1988; Li et al., 1995; Larm et al., 1995; Martinus et al., 1996). We have reported that the expression of the genes for elongation factor 1α (Wang et al., 1994) and β-actin and v-Myc (Wang & Morais, 1997) is elevated in chicken DUS3 *rho*^{0} cells. We describe here the isolation and characterization of the dual specificity kinase *MEK2* cDNA from chicken *rho*^{0} cells. In contradistinction to the above-reported observations, we found that the steady-state level of *MEK2* mRNA and that of its protein are decreased in chicken cells lacking mtDNA and in *rho*$^+$ cells rendered respiration-deficient by drugs which specifically inhibit mtDNA expression. Our studies suggest that mitochondria participate in the regulation of the mitogenic signaling pathways in chicken cells.

† This work was supported by grants from the Cancer Research Society, Inc., and the Medical Research Council of Canada. Hong Wang is a recipient of a fellowship from the Cancer Research Society, Inc.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1997.

[1] Abbreviations: Act. D, actinomycin D; CAM, chloramphenicol; CEF, chick embryo fibroblasts; cybrids, cytoplasmic hybrids; EtdBr, ethidium bromide; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKK, MAP kinase kinase (MEK); mtDNA, mitochondrial DNA; *rho*$^+$, containing mtDNA; *rho*$^-$, mtDNA deletion mutations; *rho*^{0}, devoid of mtDNA; UTR, untranslated region.

S0006-2960(97)01946-6 CCC: $14.00

MATERIALS AND METHODS

Cell Lines and Culture Conditions. DU24, a subclone of the DU249 cell line, was isolated from soft agar (Morais et al., 1988). DU249 cells were derived from a liver lesion induced in a white Leghorn chicken by the MC29 strain of avian leukosis virus (Langlois et al., 1976). BR3, a mutant isolated originally from DU24 cells mutagenized with ethyl methanesulfonate (Zinkewich-Péotti et al., 1988), is thymidine kinase-deficient, therefore resistant to 5-bromodeoxyuridine. OB2, a subclone of BR3, is further resistant to CAM and ouabain (Zinkewich-Péotti et al., 1988, 1990). These mtDNA-containing (*rho*$^+$) cells are anchorage-independent and tumorigenic in day old chicks (Zinkewich-Péotti et al., 1990, 1991). DUS3 and EB45 are mtDNA-less (*rho*^{0}) cell lines obtained in our laboratory from prolonged EtdBr-treated DU24 and BR3 cells, respectively (Morais et al., 1988; Zinkewich-Péotti et al., 1988). Both cell lines are anchorage-dependent, auxotrophic for uridine, and nontumorigenic in the wing web of 1-day-old chicks (Zinkewich-Péotti et al., 1991). Primary chick embryo fibroblasts (CEF) were prepared from 8−9-day-old white Leghorn embryos as described previously (Morais & Giguère, 1979; Desjardins et al., 1985).

OD4 and OD6 are cybrids obtained by fusion of DUS3 *rho*^{0} cells with enucleated OB2 *rho*$^+$ cells (Zinkewich-Péotti et al., 1990, 1991). The cells are resistant to CAM and sensitive to 5-bromodeoxyuridine and ouabain. CE2, CE3, and CE6 are cybrids derived from fusion of EB45 *rho*^{0} cells with enucleated CEF and are resistant to 5-bromodeoxyuridine (unpublished data). All these cybrids contain mtDNA and are prototrophic for uridine, anchorage-independent, and tumorigenic in 1-day-old chicks.

All cell lines were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum. Penicillin (100 IU/mL), streptomycin (100 μg/mL), fungizone (0.5 μg/mL), and uridine (4 μg/mL) were routinely added to the culture medium. The growth medium and the supplements were obtained from Life Technologies. Cultures were maintained at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% CO_2. Cells were passaged twice a week, and the medium was changed every other day. The population-doubling time of the *rho*^{0} and *rho*$^+$ cells is about 30 and 20 h, respectively (Morais et al., 1988). To take into account the different growth rate of the cell lines, the number of *rho*^{0} cells seeded at each passage exceeded that of the *rho*$^+$ cells by 20−25%.

Extraction of RNA and DNA. Cells at near confluence were harvested in PBS by scraping with a rubber policeman. Total cellular RNA was isolated by the variant of the guanidium isothiocyanate/CsCl method (Chirgwin et al., 1979) which includes Sarkosyl (CIBA-GEIGY) in the homogenizing buffer (Maniatis et al., 1982). Poly(A)$^+$-enriched RNA was obtained by selection on oligo(dT) immobilized on cellulose according to the manufacturer's instruction (Pharmacia Biotech Inc.). Total cellular DNA was extracted from CEF as described (Desjardins et al., 1985). The concentration of RNA and DNA solutions in water was determined by optical density. The concentration and quality of all RNA and DNA preparations were also checked by electrophoresis on agarose gels.

cDNA Libraries, Subtraction Hybridization, and DNA Sequence Analysis. Poly(A)$^+$-RNAs extracted from DUS3 *rho*^{0} and DU24 *rho*$^+$ cells were used to construct cDNA libraries in the lambda ZAPII vector from a kit from Stratagene. Subtraction hybridization was performed according to the method reported by Travis and Sutcliffe (1988). Description of the experimental procedure followed has been reported elsewhere (Wang et al., 1994, 1995).

The positive phages were purified, and the pBluescript SK$^-$ phagemid was rescued by the standard excision protocol (Stratagene). Double-stranded DNA inserts were sequenced in both directions according to the method of Sanger (1977). Sequences were compared with published data by using the program FASTA (Pearson & Lipman, 1988). Multiple alignment of the nucleotide and amino acid sequences was performed using the program packages of Corpet (1988) and Feng and Doolittle (1990), respectively.

5′-End Amplification of MEK2 mRNA. The 5′-end region of the cDNA for MEK2 was amplified from poly(A)$^+$-RNAs extracted from DUS3 *rho*^{0} cells. The first cDNA strand, obtained using a 25-mer *MEK2* antisense primer (Figure 2, nucleotides 417−393, CCATAGAAACCCACAATGTATGGGG), was ligated to a single-stranded anchor oligonucleotide provided by the 5′-AmpliFINDER RACE Kit purchased from Clontech. This cDNA was then used as a template for PCR amplification using a primer complementary to the anchor sequence and a 20-mer *MEK2* antisense primer upstream to the other one (Figure 2, nucleotides 380−360, GCAGCACCTGCAGCTCTCGG). PCR products were blunt-ended with the Klenow fragment and ligated into the pBluescript SK$^+$ vector linearized at the *Sma*I site. The structure was cloned into *Escherichia coli* XL1 strain by electroporation. Positive colonies were picked at random, the respective pBluescript phagemid was isolated, and the insert was sequenced.

Northern and Southern Blot Hybridizations. RNA preparations were analyzed by Northern and dot blot hybridizations. For Northern hybridization, 20 μg of total RNA was fractionated by size on 1.0% agarose gels under denaturing conditions (Thomas, 1980) and transferred onto Nytran membranes. For dot hybridization, 5 μg of total RNA/dot was applied onto Nytran membranes. For Southern hybridization, total cellular DNA samples (30 μg) digested with *Bam*HI, *Hin*dIII, *Kpn*I, and *Xho*I were fractionated by size on 0.7% agarose gels and transferred under alkaline conditions onto nitrocellulose membranes as described (Desjardins et al., 1985). The blots were dried, baked for 2 h at 80 °C, and hybridized with random primed [α-^{32}P]dCTP-labeled probes. The probes used were the 1.6-kb *MEK2* cDNA fragment (Figure 2), the 0.3-kb fragment of pBS77 (Figure 1), the 1.1-kb cDNA fragment of the chicken P0 ribosomal protein (Wang et al., 1995), a 0.7-kb cDNA fragment of the chicken v-Myc protein (Wang & Morais, 1997), and the 4.2-kb chicken mtDNA fragment of pMtC-10 spanning the region between ND6 and 16S rRNA (Desjardins & Morais, 1990). To ensure equal RNA loading, a 24-mer complementary to 18S rRNA (ACGGTATCTGATCGTCTTCGAACC) was ^{32}P-labeled at its 5′-end and used as a probe. Prehybridization, hybridization, and washing conditions were as recommended by the manufacturer of the membranes (Schleicher & Schuell and DuPont/NEN). Autoradiography was performed at −70 °C with intensifying screens. For reprobing, the blots were washed in boiling 0.5% SDS for 15 min. Quantification of RNAs was achieved by either laser densitometry or PhosphorImager radioactivity measurements.

In Vitro Transcription Assays. Nuclear run-on assays were done as described (Dehbi et al., 1992) with some modifications. Briefly, subconfluent cell monolayers in fresh medium were cultured overnight and collected as described above. The cells were suspended in a solution containing 15 mM NaCl, 60 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 14 mM β-mercaptoethanol, 0.5% Nonidet P-40, 0.15 mM spermine, 0.5 mM spermidine, 10% sucrose, and 15 mM Tris, pH 7.5, and lysed with a Dounce homogenizer. After centrifugation through a 30% sucrose cushion made in lysis buffer, the nuclei (2×10^7) were suspended in 100 mL of the transcription buffer [180 mM Tris-HCl, pH 8.0, 25 mM NaCl, 0.3 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 1.5 mM dithiothreitol, 700 mM $(NH_4)_2SO_4$, 8 mM $MnCl_2$, 20 mM creatine phosphate, 200 μg of creatine phosphokinase/mL, 1 mM each of ATP, CTP, and GTP] and incubated at 37 °C for 10 min in the presence of 150 mCi [α-^{32}P]UTP (3000 Ci/nmol; DuPont Canada Inc.). The ^{32}P-labeled RNAs from DU24 *rho*$^+$ and DUS3 *rho*$^-$ cell nuclei were isolated by 2-propanol−ammonium acetate precipitation. Plasmids containing the cDNA for MEK2 and v-Myc were linearized with specific restriction enzymes. After denaturation, two identical dot blots were prepared by immobilizing the DNA (10 μg/dot) onto Nytran membranes. Each filter was processed as described above and hybridized for 48 h with equal amount of counts ($1-5 \times 10^6$ cpm/mL) of the ^{32}P-labeled RNA isolated from either DU24 *rho*$^+$ or DUS3 *rho*$^-$ cell nuclei. After autoradiography, the blot probed with the ^{32}P-labeled RNA from DU24 nuclei was deprobed and rehybridized with the ^{32}P-labeled RNA from DUS3 nuclei and *vice versa*.

MEK2 mRNA Stability Analysis. Midlog to late-log-phase cell cultures were used. Cells in fresh medium were cultured overnight, and then actinomycin D (Act. D) was added. Pilot studies revealed that 5 μg/mL Act. D blocked effectively 95% [^{3}H]uridine incorporation into total RNA. Cells were harvested at various times of culture (0−24 h) by scraping and processed as indicated above to isolate total cellular RNA. Specific mRNA expression was analyzed by dot blotting (5 μg of RNA/dot). mRNA decay was determined by PhosphorImager radioactivity measurements.

Western Blot Analysis. Cells in late log phase were washed with cold PBS, harvested by scraping in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1.0% Nonidet P40, 0.25% NaDOC, 150 mM NaCl, 1 mM EGTA) containing 1 mM PMSF, 5 μg/mL each of aprotinin, leupeptin, and pepstatin, 1 mM Na_4VO_3, and 1 mM NaF. Protein content in lysates was determined according to the method of Lowry as modified for the DC protein assay (BIO-RAD). The content and quality of all protein solutions used were checked by Coomassie blue staining of samples fractionated on 10% SDS−PAGE. Total protein (30 μg) was fractionated by electrophoresis on 10% SDS−PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were exposed for 1 h at room temperature to a solution containing 1.0% BSA, 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 0.3% Tween 20 (TSBT) to prevent nonspecific absorption of antibodies and then incubated overnight at 4 °C in a 1:1000 dilution of a rabbit polyclonal anti-rat MEK2 (N-terminal residues 1−13) or anti-rat MEK1 (N-terminal residues 1−12) antiserum (Upstate Biotechnology) in the same blocking solution. Membranes were washed three times with TBST, incubated at room temperature with 0.5 μCi of ^{125}I-labeled protein A (ICN) in TSBT for 2 h, washed in TBST, and then exposed for 2 days to X-AR film (Kodak). Bands were quantified in a PhosphorImager running the ImageQuant software (Molecular Dynamic).

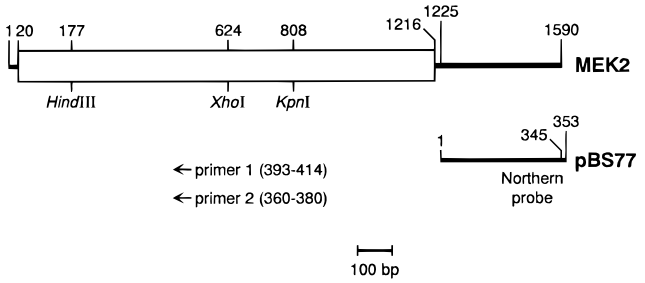

FIGURE 1: Schematic representation of the structure of chicken *MEK2* cDNA. Open box and bold lines represent the open reading frame and the 5′- and 3′-UTR, respectively. The pBS77 insert (353 nucleotides long) used as a probe in Northern studies and its relative localization on *MEK2* cDNA are indicated. The position of primers 1 and 2 (see Figure 2) used to determine the 5′-end sequence of *MEK2* mRNA by the 5′-RACE-RT-PCR procedure is shown. Sites of the major restriction enzymes used in this work are indicated.

RESULTS

Isolation and Nucleotide and Amino Acid Sequences of cDNAs Encoding the Chicken MEK2 Protein. Through the subtractive hybridization procedure used, we isolated phagemid pBS77 containing an insert 353 nucleotides long (Figure 1) with little if any homology to nucleotide sequences in the GenBank/EMBL databases. On Northern blots, this clone could hybridize to a 1.6-kb transcript in DUS3 *rho*^{0} cells, indicating that it represented a functionally active gene. To isolate a full-length cDNA, the pBS77 insert was used to screen 1×10^5 colonies from the DUS3 *rho*^{0} and DU24 *rho*$^+$ cDNA library. A total of 20 positive subclones were isolated, and the inserts in pBluescript were sequenced. Their respective size varied in length from 0.7 to 1.5 kb and contained truncated putative *MEK2* cDNAs, as inferred from similar mammalian sequences deposited with GenBank/EMBL. All *MEK2* sequences overlapped, and the longest insert, from clone pTS40, contained 1506 nucleotides (Figure 2). Compared to mammalian *MEK2* cDNAs, the chicken cDNA was found shorter at its 5′-end by about 100 nucleotides. We used a 5′-RACE-RT-PCR procedure (Figure 1) to complete the sequence, using poly(A)$^+$-RNAs isolated from DUS3 *rho*^{0} cells. DNA fragments of about 380 nucleotides long were obtained, inserted into pBluescript, and cloned. The inserts from three positive colonies were sequenced and found to vary at their 5′-end by 1 and 2 nucleotides, respectively. The longest insert added a total of 84 nucleotides to clone pTS40, and its sequence is underlined in Figure 2. The chicken *MEK2* sequence is 1590 nucleotides long, and the 5′- and 3′-UTR contain 19 and 374 bases, respectively (Figure 2). The deduced open reading frame spans the region between nucleotides 20 and 1216. The presumed polypeptide is 398 amino acids long (about 45 kDa), 2 and 3 amino acids shorter than that of the rat (Otsu et al., 1993) and human (Zheng & Guan, 1993a) and mouse (Brott et al., 1993) homologues, respectively (Table 1).

Comparisons of the chicken *MEK2* coding region with corresponding nucleotide sequences from mammals reveal a relatively high degree of sequence conservation (Table 1).

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                                  M   P   A   K   R   K   P   V   L   P   A   L   T   I   T    15
GGCGCGGCGCGGCCCGGCC ATG CCG GCC AAG AGG AAG CCG GTG CTG CCG GCC CTC ACC ATC ACT    64

 P   S   P   A   E   G   P   G   P   G   G   S   A   E   A   N   L   V   D   L    35
CCC AGC CCC GCC GAG GGA CCC GGG CCC GGC GGT TCT GCA GAG GCC AAC CTG GTG GAC CTT   124

 Q   K   K   L   E   E   L   E   L   D   E   Q   Q   K   K   R   L   E   A   F    55
CAG AAG AAG CTG GAG GAG CTG GAG CTG GAT GAG CAG CAG AAG AAG CGC TTG GAA GCT TTC   184

 L   T   Q   K   A   K   V   G   E   L   K   D   D   D   F   E   R   I   S   E    75
CTC ACT CAG AAA GCC AAA GTG GGA GAG CTG AAG GAC GAT GAC TTT GAG AGG ATC TCC GAG   244

 L   G   A   G   N   G   G   V   V   T   K   V   Q   H   K   P   S   G   L   I    95
CTG GGG GCT GGC AAC GGT GGT GTG GTC ACC AAA GTG CAA CAC AAA CCC TCA GGA CTC ATT   304

 M   A   R   K   L   I   H   L   E   I   K   P   A   I   R   N   Q   I   I   R   115
ATG GCA CGG AAG CTG ATT CAT CTA GAA ATC AAA CCA GCC ATC AGG AAT CAG ATT ATC CGA   364

 E   L   Q   V   L   H   E   C   N   S   P   Y   I   V   G   F   Y   G   A   F   135
GAG CTG CAG GTG CTG CAT GAG TGT AAT TCC CCA TAC ATT GTG GGT TTC TAT GGG GCC TTC   424

 Y   S   D   G   E   I   S   I   C   M   E   H   M   D   G   G   S   L   D   Q   155
TAC AGC GAT GGA GAG ATT TCC ATC TGC ATG GAG CAC ATG GAT GGT GGC TCC CTG GAT CAA   484

 V   L   K   E   A   K   R   I   P   E   E   I   L   G   K   V   S   I   A   V   175
GTG TTG AAA GAA GCC AAA AGA ATC CCC GAG GAA ATA CTG GGG AAA GTC AGC ATA GCA GTT   544

 L   R   G   L   A   Y   L   R   E   K   H   Q   I   M   H   R   D   V   K   P   195
CTG AGA GGT TTG GCC TAT CTG AGA GAG AAG CAC CAA ATC ATG CAC AGA GAT GTG AAG CCT   604

 S   N   I   L   V   N   S   R   G   E   I   K   L   C   D   F   G   V   S   G   215
TCC AAC ATC CTG GTT AAC TCT CGA GGA GAG ATT AAG CTG TGT GAT TTT GGG GTC AGT GGT   664

 Q   L   I   D   S   M   A   N   S   F   V   G   T   R   S   Y   M   S   P   E   235
CAA CTC ATT GAC TCC ATG GCA AAC TCT TTT GTG GGA ACT CGG TCC TAC ATG TCT CCT GAA   724

 R   L   Q   G   T   H   Y   S   V   Q   S   D   I   W   S   M   G   L   S   L   255
CGG TTG CAG GGC ACC CAT TAC TCA GTC CAG TCC GAC ATC TGG AGC ATG GGT CTG TCA CTG   784

 V   E   L   S   I   G   R   Y   P   I   P   P   P   D   S   K   E   L   E   A   275
GTG GAG CTT TCT ATT GGA AGG TAC CCC ATA CCC CCA CCA GAC TCC AAG GAA CTG GAA GCA   844

 I   F   G   R   P   V   V   D   G   A   E   G   E   S   H   S   V   S   P   W   295
ATA TTT GGC CGT CCC GTG GTG GAT GGG GCA GAG GGA GAA TCT CAC AGC GTC TCG CCG TGG   904

 A   R   P   P   G   R   P   I   S   G   H   G   M   D   S   R   P   A   M   A   315
GCC AGA CCC CCA GGA CGC CCC ATC AGT GGC CAT GGA ATG GAC AGC CGA CCT GCA ATG GCC   964

 I   F   E   L   L   D   Y   I   V   N   E   P   P   P   K   L   P   N   G   V   335
ATC TTT GAA CTG CTG GAC TAT ATC GTT AAT GAG CCA CCT CCC AAG TTG CCA AAT GGA GTT  1024

 F   T   Q   D   F   Q   E   F   V   N   K   C   L   I   K   N   P   A   E   R   355
TTC ACG CAA GAT TTC CAG GAG TTT GTA AAT AAA TGC TTA ATT AAG AAT CCA GCG GAA CGG  1084

 A   D   L   K   M   L   M   N   H   T   F   I   K   R   S   E   V   E   E   V   375
GCA GAT TTG AAG ATG CTG ATG AAT CAC ACC TTC ATC AAA CGC TCC GAA GTG GAG GAG GTG  1144

 D   F   A   G   W   L   C   K   T   L   R   L   N   Q   P   S   T   P   T   R   395
GAT TTC GCC GGC TGG CTG TGC AAA ACA CTG AGG TTA AAC CAG CCC AGC ACA CCC ACC CGT  1204

 A   A   V   *                                                                   398
GCT GCC GTG TGA TGGCCGAGCCGACTGCCTGCTGTCAGTACATCTGCCTCTGTCACCATTCCCTGTCCTTCAGCA  1279

CACAACAGAAACTGCCCTCCTTCCTTCCCCCCCACCCCCTCCAGCCCCATCCTCACCTCGCATCCCACCAGCAAAGCAA  1358

CCATCACTTGGAAACGTTAATCAGCTGCCCCCCCCCCAACCCCCCTCCCAGTCCCCTTTCCACCCCCAAGCAAACACGG  1437

GCTTGGTTTCTGTTGGTCTGGGGGTATCCTGGGCCTCTGCTCCAGCTTGGAGCTCTTGACACTTGGGAAATGTGCTGCT  1516

TATGTTAATTTTTTTTTCCTCTTTTGGTTTTTTTTTTTTGGAAACATGTTCACTTGGGTTAAAAAAAAACAAAA       1590
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FIGURE 2: Sequences of nucleotides and deduced amino acids of chicken MEK2 protein. Nucleotides are numbered in the 5′- to 3′-direction, commencing from the first nucleotide. Amino acids (one-letter code) are numbered from the first putative methionine residue in the coding region. The insert from clone pTS40 contains 1506 nucleotides and spans the region between nucleotides 85 and 1590. The sequence obtained by the 5′-RACE-RT-PCR procedure is underlined. The asterisk indicates the putative stop (TGA) codon. The nucleotide sequence has been assigned the GenBank accession L28703.

The genes evolve mainly by substitutions, and transitions outnumber transversions by 8−11%. The chicken amino acid sequence differs by 24 residues from that of the human and by 33 and 35 residues from that of mouse and rat, respectively. When changes to conservative amino acids are considered, similarities range from 98% for mouse and rat to 98.8% for human. Differences between the chicken and mammalian amino acid sequences are found mainly in the N-terminal sequence region and in the region between subdomains IX and X (Figure 3). These observations show that the MEK2 proteins have been highly conserved throughout evolution, indicating that strict functional constraints have restricted the rate of amino acid changes of vertebrate MEK2 sequences.

Total RNA and Genomic DNA Analysis. To assess the extent of expression of the chicken *MEK2* gene, total RNA samples from DUS3 *rho*^{0} and DU24 *rho*$^+$ cells were subjected to Northern analysis. We observed that the steady-state level of *MEK2* mRNA was more elevated in DU24 *rho*$^+$ than DUS3 *rho*^{0} cells by up to 3-fold (Figure 4A). Inserts from either clone pBS77 or pTS40 hybridized with a major transcript, approximately 1.6 kb long, similar in size

Table 1: Sequence Comparisons and Differences between the Coding Region of Chicken and Mammalian MEK2[a]

vertebrate	length (nt)	Nt identity (%)	Aa identity (%)	Aa similarity	number of substitutions		Aa replacement	
					Ts	Tv	Ts	Tv
chicken	1197	100						
human	1203	81.8	93.5	98.8	124	89	11	13
mouse	1206	81.0	91.0	98.0	129	92	14	19
rat	1203	80.3	90.8	98.0	136	96	15	20

[a] MEK2 from chicken contains 399 codons, including the stop codon; rat and human and mouse are 2 and 3 codons longer, respectively. Alignment of the nucleotide and amino acid sequences was performed using the program packages of Corpet (1988) and Feng and Doolittle (1990), respectively. Alignments were maximized for sequence similarity by visual inspection. Mammalian nucleotide and amino acid sequences analyzed are from human (Zheng & Guan, 1993a), mouse (Brott et al., 1993), and rat (Otsu et al., 1993). Abbreviations: Nt, nucleotides; Aa, amino acids; Ts, transitions; Tv, transversions.

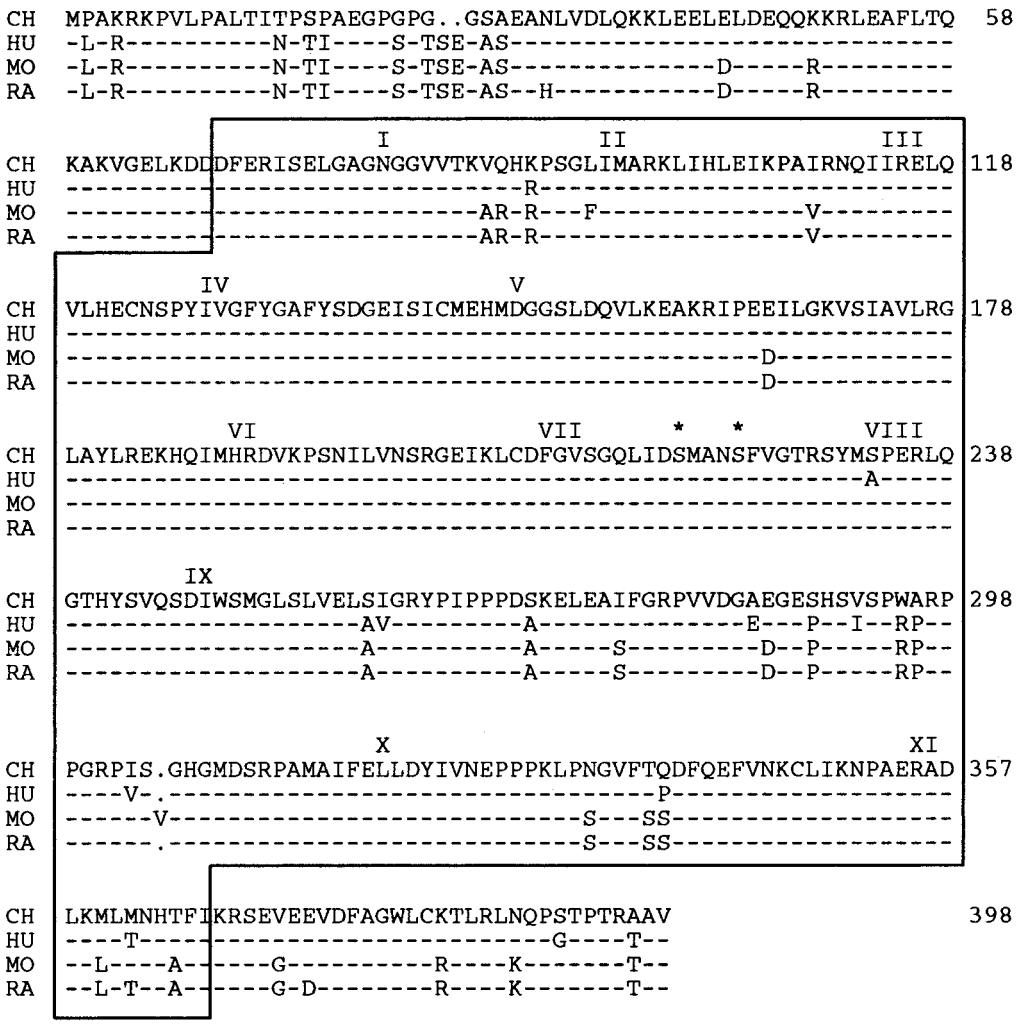

FIGURE 3: Comparison of chicken MEK2 amino acid sequence with similar proteins from mammals. Species compared: CH, chicken; HU, human (Zheng & Guan, 1993a); MO, mouse (Brott et al., 1993); RA, rat (Otsu et al., 1993). The protein sequences are presented in single-letter code. The chicken sequence is shown in full and numbered as in Figure 2. Residues identical to chicken are indicated by dashes and gaps introduced to optimize alignment by dots. The boxed area encompasses the catalytic domain of the MEK2 proteins, and the subdomains are marked with roman numbers as proposed by Hanks et al. (1988). The phosphorylated sites in actived MEK2 proteins are indicated by asterisks.

to human and rat *MEK2* mRNAs (Zheng & Guan, 1993a; Otsu et al., 1993). In addition, a minor species of 3.4 kb long was consistently detected in both cell lines. The absence of mtDNA transcription products in DUS3 *rho*^{0} cells was confirmed using a chicken mtDNA probe that recognizes 12S and 16S mitochondrial rRNAs (Desjardins et al., 1989). We used acidic ribosomal phosphoprotein P0 as an internal control for RNA loading because its steady-state expression is similar in DUS3 *rho*^{0} and DU24 *rho*$^+$ cell lines (Wang et al., 1995).

To determine whether the chicken genome contains one or many copies of the gene for MEK2, total DNA samples from primary chick embryo fibroblasts were digested with different restriction enzymes and hybridized with the insert from pTS40. Only two hybridizing DNA fragments were detected in *Kpn*I-digested DNA (Figure 4B), consistent with the presence of one *Kpn*I site in chicken *MEK2* cDNA (Figure 1). Thus it appears that *MEK2* is a single-copy gene in chicken, in contrast to the situation in rat where multiple copies of the same gene and retrospeudogenes exist (Otsu

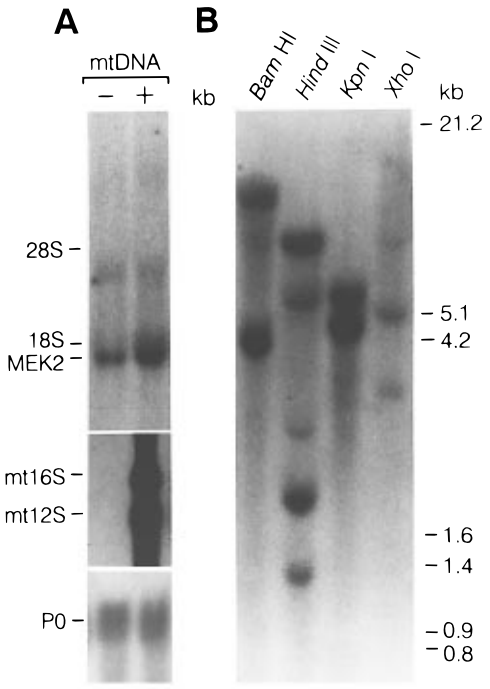

FIGURE 4: Relative steady-state level of *MEK2* mRNAs in chicken cells with (+) or without (−) mtDNA and Southern blot analysis of chicken genomic DNA. (A) A 20-μg sample of total cellular RNA from DU24 (mtDNA$^+$) and DUS3 (mtDNA$^-$) cells was fractionated by size on a 1% agarose−2 M formaldehyde gel and transferred onto a Nytran membrane. The resulting filter was then hybridized with the random primed ^{32}P-labeled *MEK2* 3′-UTR recovered from pBS77. The filter was deprobed and rehybridized successively with the cDNA fragment of the chicken P0 ribosomal protein (Wang et al., 1995) to ensure equal RNA loading and the chicken mtDNA fragment spanning the region between ND6 and 16S rRNA (Desjardins et al., 1989) to exemplify the absence of mtDNA transcripts in DUS3 *rho*^{0} cells. The size of hybridizing fragments is indicated on the right. (B) Samples of 30 μg of total cellular DNA from primary chicken embryo fibroblasts were digested with *Bam*HI, *Hin*dIII, *Kpn*I, and *Xho*I, respectively. After fractionation by size on a 0.7% agarose gel, the digested materials were transferred onto a nitrocellulose membrane and then hybridized with the 1.6-kb *MEK2* cDNA fragment (Figure 2). The size of DNA marker fragments is indicated on the right.

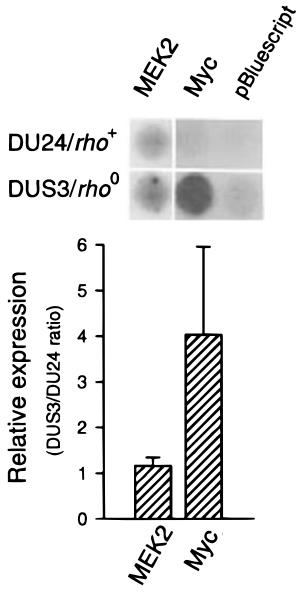

FIGURE 5: Nuclear run-on transcription assays. (Top) Representative run-on transcription assay autoradiograms. Nuclei from chicken cells DU24 *rho*$^+$ and DUS3 *rho*^{0} were isolated by differential centrifugation, and *in vitro* transcription was allowed to resume in the presence of [α-^{32}P]UTP. Equal amounts of counts (cpm) of ^{32}P-labeled, *in vitro* transcribed RNA probes from DU24 *rho*$^+$ and DUS3 *rho*^{0} nuclei were hybridized to two identical membranes with immobilized linearized plasmid DNAs (10 μg) inserting the cDNA of the genes for MEK2 and v-Myc. After autoradiography, each filter was deprobed and rehybridized with ^{32}P-labeled RNAs isolated from the other cell line and *vice versa*. (Bottom) Densitometric measurement analysis of three independent experiments. Values are the mean ± SD.

et al., 1993). The two transcripts observed by Northern blot analysis likely result from differences in mRNA splicing or processing.

MEK2 mRNA Stability Is Altered in mtDNA-Less Cells. Downregulation of the gene for MEK2 in DUS3 *rho*^{0} cells could result from a decrease in its transcriptional rate, an increased rate of mRNA degradation (i.e., decreased stability), or both mechanisms occurring simultaneously. To investigate these possibilities, we first performed run-on transcription assays on nuclei isolated from DU24 *rho*$^+$ and DUS3 *rho*^{0} cells and compared the transcriptional rate of the gene for MEK2 to that of the gene for v-Myc, whose rate of transcription is increased in *rho*^{0} versus *rho*$^+$ cells (Wang & Morais, 1997). The results obtained indicated that the transcriptional rate for *MEK2* is similar in cells with and without mtDNA (Figure 5). To determine if increased rate of *MEK2* mRNA degradation could account for decreased expression of the gene in DUS3 *rho*^{0} cells, we then exposed DU24 *rho*$^+$ and DUS3 *rho*^{0} cells to Act. D and analyzed as a function of time the steady-state level of *MEK2* mRNAs. Under these experimental conditions, the half-life of *MEK2* mRNAs was estimated to be 11 h in DUS3 *rho*^{0} cells and 26 h in DU24 *rho*$^+$ cells (Figure 6): under the same experimental conditions the half-life of the *v-myc* transcript remained the same in both *rho*^{0} and *rho*$^+$ cells (Wang & Morais, 1997). Taken together, these data suggest that decreased expression of *MEK2* in DUS3 *rho*^{0} versus DU24 *rho*$^+$ cells results, at least in part, from destabilization of the *MEK2* transcript in *rho*^{0} cells.

Effect of Ethidium Bromide and Chloramphenicol on MEK2 Gene Expression. To determine whether the inhibition of mtDNA expression could induce changes in *MEK2* gene expression similar to that seen in DUS3 *rho*^{0} cells, parental DU24 *rho*$^+$ cells were treated with either EtdBr, a specific inhibitor of mtDNA replication and transcription in cultured avian cells (Desjardins et al., 1985, 1989), or CAM, an antibiotic which blocks specifically translation on mitoribosomes in cultured vertebrate cells (Schatz & Mason, 1974; Morais, 1980). As expected, EtdBr strongly inhibited the expression of the mtDNA genes, as indicated by the intensity of the signals given by the mtDNA probe pMtC-10 in cells exposed to the drug for 1, 3 and 6 days (Figure 7); CAM had little if any effect on mtDNA expression during this period. In the presence of one or the other drug, the relative expression of *MEK2* decreased as a function of time, up to 50%.

MEK2 Expression in Cytoplasmic Hybrids. To explore further the effect of mtDNA expression on MEK2 gene expression, we investigated the effect of repopulating *rho*^{0} cells with mtDNA-containing mitochondria on the steady-state level of *MEK2* mRNAs. Cybrids established previously by the fusion of DUS3 *rho*^{0} cells with enucleated parental OB2 cells (OD4 and OD6) and EB45 *rho*^{0} with enucleated CEF (CE2, CE3, and CE6) were analyzed. As shown in

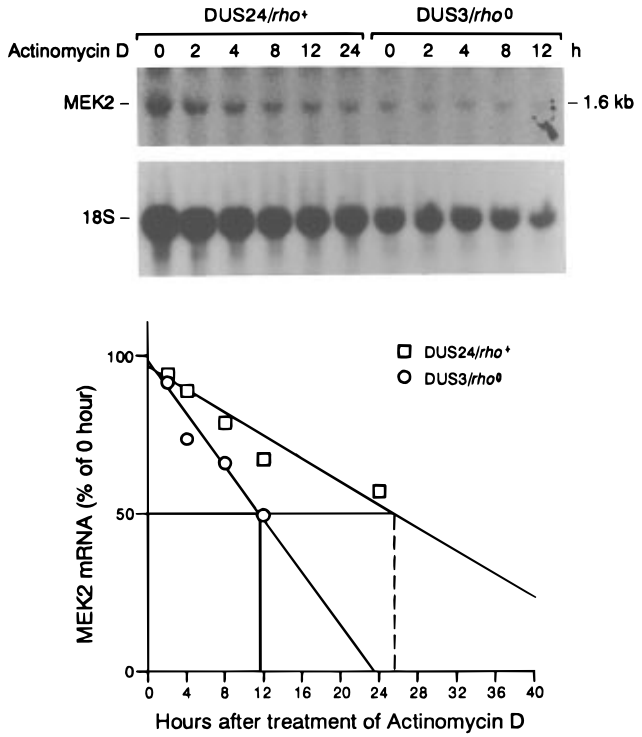

FIGURE 6: Half-life of *MEK2* mRNAs. Total cellular RNA from DU24 *rho*$^+$ and DUS3 *rho*^{0} cells was extracted at the indicated times after Act. D addition in the culture medium. Northern blot analysis was performed with 20- and 10-μg samples of total RNA/lane for DU24 *rho*$^+$ and DUS3 *rho*^{0} cells, respectively. The experimental procedure followed is described in the legend of Figure 4 and the Materials and Methods section. The corrected signal density was then plotted as a percentage of zeroth-hour value against time.

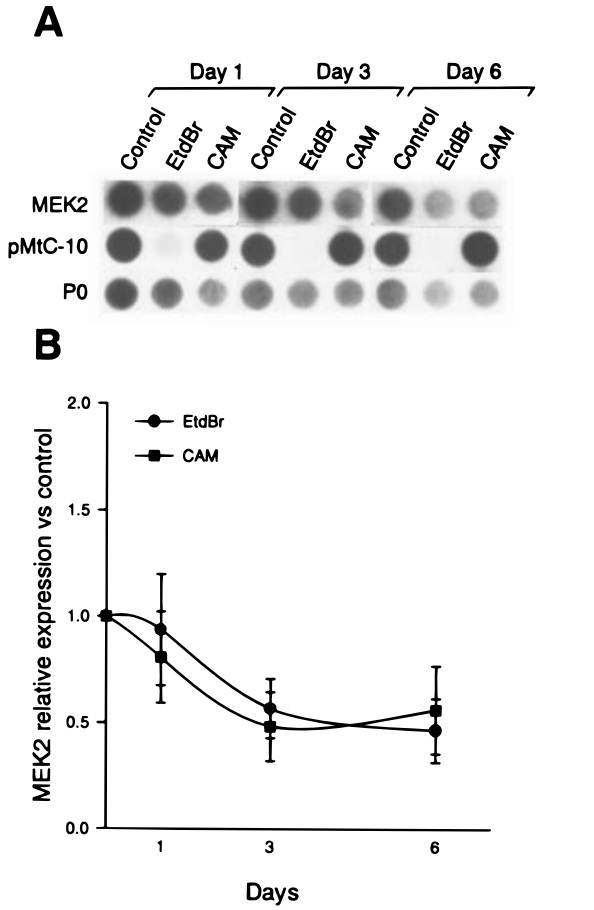

FIGURE 7: Effect of EtdBr and CAM on *MEK2* expression. (A) Total cellular RNA was extracted from DU24 *rho*$^+$ cells treated with EtdBr (0.1 μg/mL) or CAM (100 μg/mL) for 1, 3, and 6 days; 5 μg of total RNA was blotted onto a Nytran membrane and then hybridized with the ^{32}P-labeled 1.6-kb *MEK2* cDNA probe (Figure 2). The filter was deprobed and rehybridized successively with the mtDNA fragment recovered from pMtC-10 (Desjardins & Morais, 1990) and the chicken P0 ribosomal protein cDNA used as an internal control (Wang et al., 1995). (B) Densitometric measurement analysis of three independent experiments. MEK2 signals were corrected by P0 signals and plotted as a relative expression (control = 1.0). Values are the mean ± SD.

Figure 8, the steady-state level of *MEK2* mRNAs in cybrids is similar to those in parental mtDNA-containing cells, strongly supporting the idea that mitochondria are involved in the expression of the gene for MEK2 in cultured chicken DU24 cells.

MEK1 and MEK2 Protein Content in mtDNA-Less Cells and in Parental Cells Exposed to Chloramphenicol and Ethidium Bromide. We performed Western blot analysis to determine whether the MEK2 protein content correlated with that of the *MEK2* transcripts in cells with and without mtDNA and in parental *rho*$^+$ cells treated during 6 days with EtdBr and CAM. We found that the MEK2 protein content is indeed decreased in DUS3 *rho*^{0} cells as well as in parental DU24 *rho*$^+$ cells treated with EtdBr and CAM (Figure 9). We then decided to examine the presence of the MEK1 protein, a kinase related to MEK2 (Otsu et al., 1993). In sharp contrast to MEK2, the MEK1 protein content remained the same in DUS3 *rho*^{0} cells and in parental *rho*$^+$ cells exposed to EtdBr and CAM, indicating that the two MEK isoforms are differently regulated in chicken cells when mtDNA expression is impaired.

DISCUSSION

MAPK/ERK kinase (MEK) or MKK (for MAP kinase kinase) (Wu et al., 1993; Davis, 1993) is a family of dual specificity kinases that phosphorylate and activate specific mitogen-activated protein kinases (MAPKs) through diverse signal transduction pathways (Davis, 1993; Pelech & Sanghera, 1992; Crews & Erikson, 1993; Egan & Weinberg, 1993; Mordret, 1993). Members of the MEK family receive signals from different upstream signaling pathways and transmit them to the MAPKs, which direct the expression of genes involved in cell division, differentiation, and compensation for alterations in the extracellular milieu.

Six members of the MEK family have been identified so far in vertebrate cells and at least five MEK/MAPK pathways defined (Davis, 1994). Among the MKK, MEK1 (Otsu et al., 1993; Crews et al., 1992) and MEK2 (Otsu et al., 1993; Brott et al., 1993) are the only two identified MAPK activators in mitogenic growth factor signal transduction pathways. These MEKs display an extremely high substrate selectivity for the extracellular signal-regulated kinases ERK1 and ERK2 (Boulton et al., 1991; Seger et al., 1991; Robbins et al., 1993), which they activate by phosphorylation on conserved neighboring threonine and tyrosine residues separated by a single glutamic acid residue, the TEY site (Payne et al., 1991; Posada & Cooper, 1992). The MEK/ERK cascade has been shown to play a pivotal role in diverse biological processes including proliferation and transformation (Cobb et al., 1994).

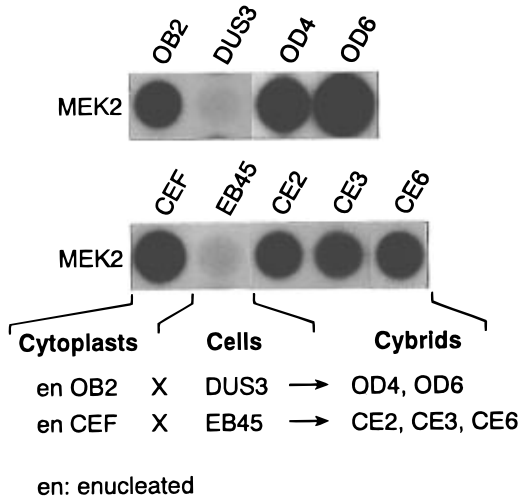

FIGURE 8: Dot blot analysis of *MEK2* mRNAs in cybrids. Representative autoradiograms of *MEK2* expression in cells with (OB2 and CEF) and without (DUS3 and EB45) mtDNA and in cybrids (OD4, OD6, CE2, CE3, and CE6); 5 μg of total RNA was blotted onto a Nytran membrane and hybridized with the ^{32}P-labeled chicken *MEK2* cDNA probe (Figure 2). Clones from enOB2 × DUS3 were first selected in Ham's F12 medium containing dialyzed serum, hypoxanthine, amethopterin, and thymidine, and CAM (100 μg/mL), ring-cloned, propagated, and individually tested for resistance to 100 μM ouabain (Zinkewich-Péotti et al., 1990). Clones from enCEF × EB45 were selected in medium containing dialyzed serum and 5-bromodeoxyuridine (100 μg/mL).

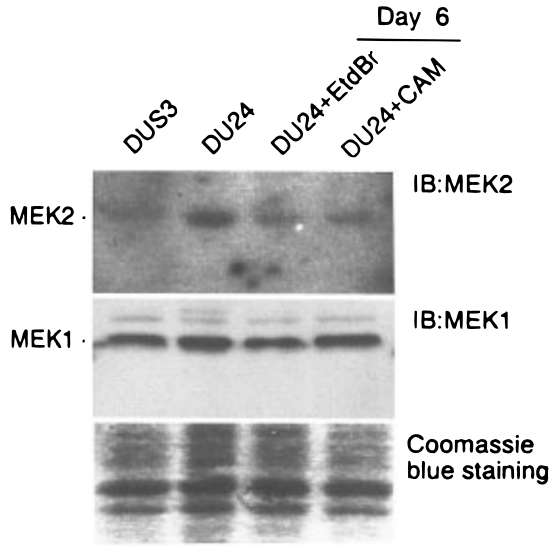

FIGURE 9: MEK2 and MEK1 protein content. Whole cell lysates were prepared from DUS3 *rho*^{0} and DU24 *rho*$^+$ cells treated with CAM (100 μg/mL) or EtdBr (0.1 μg/mL) for 6 days; 30-μg samples of total protein were analyzed by Western blotting with rabbit anti-rat MEK2 (N-terminal residues 1−13) and anti-rat MEK1 (N-terminal residues 1−12) polyclonal antibodies. Coomassie blue staining is shown to illustrate equivalent loading of the proteins.

In this paper, we describe the cloning and characterization of chicken *MEK2* cDNA and the regulation of the gene in a chicken mtDNA-less cell system. Two mRNA species (1.6 and 3.4 kb long) that hybridized with the 3′-untranslated region of *MEK2* cDNA were detected by Northern blot analysis in cells with or without mtDNA. The two forms of mRNA are most likely derived by alternative splicing of the primary *MEK2* transcript, since Southern analysis suggested the presence of a single *MEK2* gene in CEF.

Northern and Western blot analyses demonstrated that the cellular content of *MEK2* mRNA and MEK2 protein is decreased in chicken mtDNA-less cells. This situation can be reproduced by exposing parental mtDNA-containing cells to the mitochondrial replication and transcription inhibitor EtdBr and to the translation inhibitor CAM. Conversely, MEK2 expression is recovered in mtDNA-less cells after their fusion with mtDNA-containing cytoplasts prepared from either transformed or normal cultured cells. We found that the transcriptional rate of the *MEK2* gene remained unchanged in mtDNA-less cells, while the *MEK2* mRNA half-life decreased from 26 to 11 h, suggesting that mRNA destabilization contributes significantly to decreased expression of the *MEK2* gene in chicken mtDNA-less cells.

Messager RNA stability plays a key role in many regulatory processes, including those related to cell cycle regulation, tumorigenicity, and development. For exemple, degradation of histone mRNAs is strongly activated when DNA synthesis stops (Stein et al., 1994). In human breast cancer cells, histone H4 and cyclin mRNAs are strongly stabilized relative to normal cells (Keyomarsi & Pardee, 1993). Mutations that lead to the stabilization of *c-fos* and *c-myc* mRNAs contribute to cell transformation and tumorigenicity (Schiavi et al., 1992). Although the molecular mechanisms involved in differential rates of mRNA degradation are still poorly understood in vertebrates, *cis*- and *trans*-acting elements, such as specific sequences or secondary structures of the mRNA, and nucleases have been found to modulate the rate of mRNA decay (Beelman & Parker, 1995; Ross, 1995; Surdej et al., 1994). We have searched for the presence of the pentose sequence AUUUA in the 3′-untranslated region (UTR) of the message for *MEK2*. This sequence occurs in a variable number in the 3′-UTR of many short-lived mRNAs (Wilson & Treisman, 1988; Schuler & Cole, 1988; Kruys et al., 1989) and has been shown to destabilize mRNAs in cultured cells (Savant-Bhonsale & Cleveland, 1992). Although A+T-rich nucleotide stretches are found in *MEK2* 3′-UTR (Figure 2), the sequence motif ATTTA is not present, suggesting that a different molecular mechanism participates to the instability of *MEK2* mRNA in chicken mtDNA-less cells.

The present findings suggest that mitochondria are involved in the regulation of the stability of *MEK2*, and perhaps other mRNAs, in DU24 chicken cells. We have previously reported that transcriptional upregulation contributed to elevated expression of a number of nuclear genes in chicken mtDNA-less cells (Wang et al., 1994; Wang & Morais, 1997). Altogether, these findings lead to further questions about the role of mitochondria in regulation of the expression of non-mitochondrial genes in chicken and other vertebrates: Specifically, what is the nature of information produced by mitochondria? How does it control the expression of nuclear genes? What is the biological significance of this regulation? Recent observations indicate that short peptides, derived from mtDNA-encoded proteins, and the large ribosomal RNA (16S) are exported outside the mitochondrion (Loveland et al., 1990; Kobayashi et al., 1993; Ding et al., 1994). Whether these or other mtDNA-derived products could behave like intracellular modulators of nuclear gene expression remains to be established. On the other hand, and given the limited information content of vertebrate mtDNAs, it may be that differential nuclear gene expression could result from metabolic changes induced in cells during their adaptation to chronic mitochondrial dysfunctions (Grivell, 1989; Kuiper et al., 1988). Whatever the exact molecular

mechanism(s) involved in this process, the present observations, and those reported previously (Liao et al., 1991; Rothermel et al., 1995; Li et al., 1995; Larm et al., 1995; Wang & Morais, 1997), indicate that eukaryotes, from yeast to vertebrates, respond to altered mitochondrial functions by modulating the expression of nuclear genes. The extent to which the state of differentiation and/or the nuclear genetic content of the cells modulates the nuclear response to inhibition of mtDNA expression in vertebrates remains to be determined (Chomyn et al., 1994; Wang & Morais, 1997).

In contrast to MEK2, the protein content of MEK1 remains unchanged in cells depleted of mtDNA and in cells treated with inhibitors of the mitochondrial macromolecular-synthesizing systems, suggesting that both kinases are differently regulated in chicken cells. It has been reported that constitutively activated *MEK1* transformed rodent cells, which grew efficiently in soft agar, and are highly tumorigenic in nude mice (Cowley et al., 1994; Brunet et al., 1994; Mansour et al., 1994). Although speculative, impaired activity of MEK2 in chicken mtDNA-less cells may be relevant to the fact that these cells have lost their tumorigenic capacity *in vivo* and *in vitro* but recovered these properties and basal level of *MEK2* expression (Figure 8) when fused with cytoplasts from enucleated chicken mtDNA-containing cells (Zinkewich-Péotti et al., 1990, 1991). This is consistent with the observation that recombinant human MEK2 has an activity approximately 7 times higher than that of MEK1 toward the extracellular signal-regulated kinases ERK1 and ERK2 (Zheng & Guan, 1993b). Future studies will determine if the MEK/ERK module is downregulated in chicken mtDNA-less cells and in human *rho*^{0} cells showing diminished tumorigenic capacity (Hayashi et al., 1992; Morais et al., 1994, 1996) and whether forced expression of *MEK2* in native or constitutively active form restores tumorigenic potential to *rho*^{0} cells.

ACKNOWLEDGMENT

We thank Louise Cournoyer, Manon Parent, and Nathalie Boels for technical assistance, and Drs. M.-E. Lee and N. Sibinga for critically reviewing this manuscript.

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BI971946X