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IN VITRO TRANSLATION OF CANINE MITOCHONDRIAL CREATINE KINASE MESSENGER RNA

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SUMMARY. The cell-free translation products of mRNA from canine myocardium were immunoprecipitated using antiserum specific for either the MM or mitochondrial creatine kinase subunit. The two subunits were shown to be encoded by the nuclear genome and translated from separate mRNAs. The mitochondrial subunit was translated as a polypeptide with a molecular weight approximately 6,000 greater than the mature form of the enzyme. In contrast, the M-subunit was translated as a polypeptide having a molecular weight identical to that of the mature cytosolic M-subunit. It is assumed that the mitochondrial subunit precursor must be proteolytically processed during translocation from the cytoplasm into mitochondria.

ATP: creatine N-phosphotransferase (E.C. No. 2.7.3.2), commonly referred to as creatine kinase, catalyses the reversible transfer of a phosphate residue between ATP and creatine. Creatine kinase is a dimeric molecule with a molecular weight of approximately 84,000. The cytoplasm of mammalian tissues contain two different kinds of subunits: muscle type (M) and brain type (B) both of which have molecular weights of approximately 42,000. Three isoenzymes (MMCK, MBCK and BBCK) are produced by various combinations of the two subunits. Mitochondria from mammalian tissues have been shown to contain a CK which differs immunologically (5), in electrophoretic mobility and amino acid content (1, 2, 3, 4) from the cytosolic isoenzymes. Because of its location on the outer surface of the inner mitochondrial membrane (3, 6, 7) and its higher substrate affinity for ATP than the cytosolic forms, it is assumed that mitochondrial CK plays a major role in the transfer of energy to the cytoplasm

Abbreviations: CK = creatine kinase; SDS = sodium dodecylsulphate.

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(3, 8). It is unknown whether mitochondrial CK is encoded by the nuclear or mitochondrial genome.

Recent studies of eukaryotic mitochondrial protein biosynthesis (9, 10, 11, 12, 13, 14, 15) have demonstrated that most mitochondrial proteins are encoded on the nuclear genome. The mRNAs of these mitochondrial proteins are translated in the cytoplasmic compartment and encode, in most instances, higher molecular weight precursor polypeptides. Following translation, these larger precursors are released into the cytoplasm and subsequently translocated across mitochondrial membranes. This post-translational translocation is accompanied by proteolytic processing, resulting in the mature mitochondrial protein subunit.

As a first step in the study of the biosynthesis of CK isoenzymes, we used an *in vitro* cell-free system to examine the primary translation products of canine myocardial mRNA. Our results indicate that both the M and mitochondrial subunits are encoded by the nuclear genome and that the *in vitro* translated mitochondrial CK subunit was approximately 6,000 greater in molecular weight than the mature mitochondrial or M-CK subunits. We assume that, *in vivo*, pre-mitochondrial CK is proteolytically processed into the mature mitochondrial CK subunit during translocation from the cytoplasm into mitochondria.

MATERIALS AND METHODS

Preparation of Total Cellular RNA from Canine Myocardium: Myocardium was removed from adult dogs and immediately used for RNA extraction or was frozen in liquid nitrogen and stored at -70°C for future use. Twenty g of myocardium was homogenized in 250 ml of 4 M of guanidinium thiocyanate buffer and RNA purification was done according to the procedure of Chirgwin *et al* (16). To insure maximal myocardial RNA recovery, all ethanol precipitations were incubated for 16 hours at -20°C. Because of the length of time required to isolate RNA by this procedure, we also used the guanidinium thiocyanate-cesium chloride technique (17). RNA prepared by either procedure had a ratio of absorbance at 260 nm/280 nm of 1.7-1.9.

[^{35}S]methionine: [^{35}S]Methionine was obtained from New England Nuclear.

Purification of Creatine Kinase Isoenzymes: Creatine kinase isoenzyme MM was purified as previously described (18). Mitochondrial creatine kinase was purified by the procedure of Roberts and Grace (5).

Development of Specific Antiserum to Creatine Kinase: Specific antiserum to the M subunit of canine creatine kinase was prepared in rabbits and characterized as previously described (19, 20). Specific antiserum to mitochondrial

creatine kinase was produced and characterized as described by Roberts and Grace (5). The antisera were examined by immunodiffusion and a soluble phase double antibody radioimmunoassay and were shown to be subunit specific. No cross-reactivity between subunits was observed (5).

Protein Determinations: Proteins were determined by the method of Lowry et al (21).

Immunoprecipitation and Analysis of the Translation Products of Canine Myocardial mRNA: Total cellular RNA was translated in a cell-free system derived from wheat germ as described by Gordon et al (22). The concentration of RNA used in the reaction mixture was 400 µg/ml. The amount of [^{35}S]methionine incorporation into protein was determined by trichloroacetic acid precipitation (22). Immunoprecipitation of translation products was performed using antiserum specific for either the M or mitochondrial subunit of CK by the procedure of Conboy and Rosenberg (11). Competitive immunoprecipitation was done by addition of 50 µg of MMCK or 30 µg of mitochondrial CK to the translation system immediately before the addition of specific antiserum. Immunoprecipitates were applied to 10.8% polyacrylamide gels containing SDS prepared according to Laemmli (25). Following electrophoresis, the dried gels were subjected to fluorography at -70°C.

RESULTS AND DISCUSSION

In order to determine whether the M and mitochondrial subunits are both encoded in the nuclear genome, we used an in vitro translation system to translate canine myocardial RNA. After translation in a wheat germ cell-free system in the presence of [^{35}S]methionine, immunoprecipitation with subunit monospecific antiserum was performed (Figure 1). The translation product immunoprecipitated by M-CK specific antiserum was a single polypeptide with Mr = 42,000 which was identical with unlabeled M-CK in electrophoretic mobility. This finding confirms those of several previous studies, demonstrating the apparent identity of the primary product of M-CK mRNA with the mature protein subunit (25, 26). On the other hand, the mitochondrial CK immunoprecipitate consisted of two major polypeptides. The lower band was tentatively identified as actin because of its molecular weight and the well recognized ability of actin to react in a nonspecific manner with IgG (27). The higher molecular weight band (Mr = 48,000) was assumed to be the mitochondrial CK subunit. To confirm that the 48,000 molecular weight polypeptide was the mitochondrial creatine kinase subunit precursor, a competition immunoprecipitation experiment was performed (Figure 2). Mature unlabeled mitochondrial CK inhibited the immunoprecipitation of the precursor polypeptide by specific antiserum (lanes E and F). In contrast, the M-CK subunit was trans-

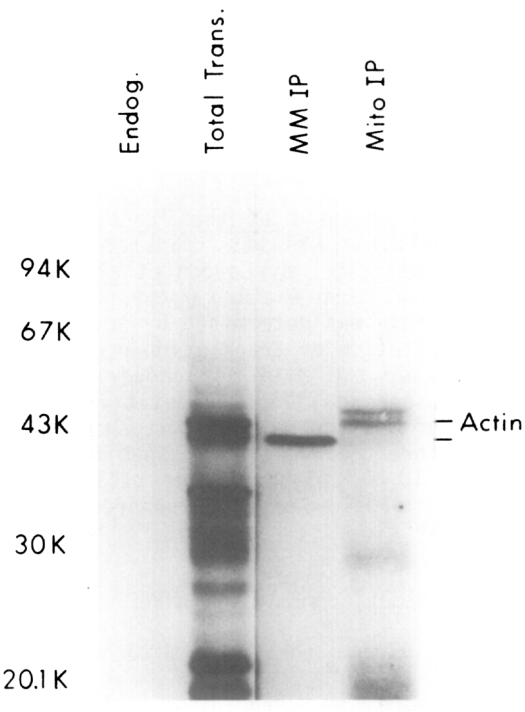

Figure 1: Specific immunoprecipitation of creatine kinase subunit translation products from a cell-free wheat germ system programmed with canine myocardial RNA. Shown are fluorographs of dried 10.8% SDS-polyacrylamide gels. Endogenous translation products and total translation are so labeled and were exposed to x-ray film for 18 h. The immunoprecipitate of a 50 µl reaction mixture with antiserum specific for the M subunit is labeled MM IP and was exposed to x-ray film for 48 h. The immunoprecipitate of a 50 µl reaction mixture by antiserum specific for the mitochondrial subunit is labeled Mito IP and was exposed for 120 h. The actin contaminate of this immunoprecipitate is labeled. The lower black mark indicates the distance migrated by unlabeled M subunit electrophoresed on the same gel. All polypeptides were labeled with [^{35}S]methionine. Molecular weight markers are phosphorylase B (Mr = 94,000); bovine serum albumin (Mr = 67,000); ovalbumin (Mr = 43,000); carbonic anhydrase (Mr = 30,000); soybean trypsin inhibitor (Mr = 20,100) and α-lactalbumin (Mr = 14,400).

lated as a polypeptide with a molecular weight identical (as indicated by arrow) to that of the M subunit isolated from myocardium (lane C). Actin contamination of the mitochondrial subunit immunoprecipitation as in the previous experiment did not occur. The reason for this is not clear to us, but may be due to the fact that a different procedure was used for RNA purification.

In the present study, we used RNA isolated from canine myocardium and antisera specific for either the M-CK or mitochondrial CK to examine the *in vitro* cell-free translation products of canine myocardial mRNA for the

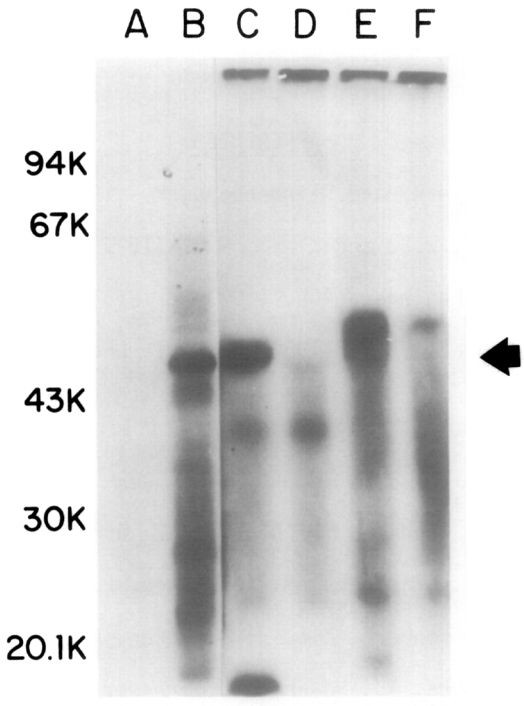

Figure 2: Competitive immunoprecipitation of creatine kinase subunit translation products from a cell-free wheat germ system programmed with canine myocardial RNA. Shown are fluorographs of dried 10.8% SDS-polyacrylamide gels exposed to x-ray film for 24 h (lanes A and B) or 48 h (lanes C-F). Lane A is the endogenous translation control and lane B is the total translation products. The immunoprecipitates of a 50 µl reaction mixture by antiserum specific for the M subunit is shown in lane C. Lane D shows the immunoprecipitate from an identical reaction mixture to which 50 µg of purified M subunit was added immediately before the specific antiserum. Lane E shows the immunoprecipitate of a 100 µl reaction mixture by antiserum specific for the mitochondrial subunit. Lane F shows the immunoprecipitate from an identical 100 µl reaction mixture to which 30 µg of purified mature mitochondrial subunit was added. The arrow indicates the migration of unlabeled M subunit on the same gel. All polypeptides were labeled with [^{35}S]methionine. The molecular weight standards are as described in Figure 1.

presence of the two CK subunits. We showed that mitochondrial and MMCK are encoded by the nuclear genome and the mitochondrial CK subunit was translated as a precursor polypeptide with a molecular weight approximately 6,000 greater than the mature form of the enzyme. It is assumed that the precursor is proteolytically cleaved during transport into the mitochondria to produce the mature form of the enzyme. In contrast, the M-CK subunit was translated from a different mRNA as a polypeptide of identical molecular weight to the mature cytoplasmic M subunit.

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