Opioid Peptide Gene Expression Primes Cardiogenesis in Embryonal Pluripotent Stem Cells

Carlo Ventura, Margherita Maioli

Abstract—Zinc finger–containing transcription factor GATA-4 and homeodomain Nkx-2.5 govern crucial developmental fates and have been found to promote cardiogenesis in embryonic cells exposed to the differentiating agent DMSO. Nevertheless, intracellular activators of these transcription factors are largely unknown. In this study, pluripotent P19 cells expressed the prodynorphin gene, an opioid gene encoding for the dynorphin family of opioid peptides. P19 cells were also able to synthesize and secrete dynorphin B, a biologically active end product of the prodynorphin gene. DMSO-primed GATA-4 and Nkx-2.5 gene expression was preceded by a marked increase in prodynorphin gene expression and dynorphin B synthesis and secretion. The DMSO effect occurred at the transcriptional level. In the absence of DMSO, dynorphin B triggered GATA-4 and Nkx-2.5 gene expression and led to the appearance of both α-myosin heavy chain and myosin light chain-2V transcripts, two markers of cardiac differentiation. Moreover, dynorphin B–exposed cells were positively stained in the presence of MF 20, a mouse monoclonal antibody raised against the α-myosin heavy chain. Opioid receptor antagonism and inhibition of opioid gene expression by a prodynorphin antisense phosphorothioate oligonucleotide blocked DMSO-induced cardiogenesis, suggesting an autocrine role of an opioid gene in developmental decisions. (Circ Res. 2000;87:189-194.)

Key Words: opioid gene expression ■ myocardial development ■ transcription factors ■ stem cells

Heart development is one of the first morphogenetic events occurring in the embryo and is a complex process involving cell proliferation and differentiation as well as tissue organization into a specific architecture. The analysis of myocardial differentiation lies in determining how a small number of tissue-restricted transcription factors can establish a complex pattern of developmental and tissue-specific gene expression. It is now recognized that Nkx-2.5, a vertebrate homologue of the Drosophila tinman gene,1 is essential for the specification of myocardial progenitors in the fly2 and is also involved in heart looping in mice.3 Recently, mutations in the transcription factor Nkx-2.5 have been shown to cause congenital heart diseases, even in humans,4 and impair normal heart architecture by affecting diverse cardiac developmental pathways in a wide population of cardiopathic patients.5 GATA-4, a member of the zinc finger–containing GATA factors,6 also plays a key role in cardiac muscle development and has been found to govern the expression of several cardiac-specific genes, including α-myosin heavy chain (MHC), cardiac troponin C, and atrial natriuretic peptide.6 The availability of pluripotent embryonal stem (ES) cells now provides a valuable tool for the molecular dissection of developmental events. P19 is a line of pluripotent murine ES cells that can be differentiated into multiple cell types by cellular aggregation in the presence of differentiating agents and emulates the molecular and morphological events occurring during early embryonic development.7 P19 cells treated with retinoic acid differentiate into various neuroectodermal derivatives, whereas cells aggregated in the presence of DMSO differentiate into cardiac and skeletal muscle along with other mesodermal and endodermal cell types.7 The resulting cardiomyocytes have been found to be embryonic in nature and express both GATA-4 and Nkx-2.5 genes.8 In P19 cells, inhibition of GATA-4 expression blocked DMSO-induced cardiogenesis,9 whereas transfection of the Nkx-2.5 gene led to the appearance of a myocardial lineage in the absence of DMSO.8 These findings indicate that the establishment of a cardiac phenotype proceeds with similar molecular patterning in P19 cells and in the in vivo environment and suggest that these cells should be considered as an in vitro model recapitulating the molecular plight that is impacting the process of cardiogenesis in the developing embryo. The identification of intracellular growth regulators capable of priming cardiogenesis is now a crucial issue. Increasing experimental evidence indicates that the myocardial cell may act as a source for peptides, playing a crucial role in signal transduction mechanisms. We have shown that cardiac myocytes express the prodynorphin gene and are able to synthesize and secrete dynorphin B,10 a natural κ-opioid receptor agonist. Stimulation of these receptors modified both
cytosolic Ca\textsuperscript{2+} and pH homeostasis and remarkably affected myocardial contractility.\textsuperscript{11,12} It is now established that opioid peptides can also act as growth regulators in a wide variety of normal and malignant tissues.\textsuperscript{13–15} In our previous investigations, prodynorphin gene and dynorphin B expression were enhanced in myocytes isolated from BIO 14.6 Syrian hamsters,\textsuperscript{16} an experimental model of hereditary cardiomyopathy exhibiting remarkable changes in myocyte architecture, growth, and differentiation. In this model, the overexpression of the prodynorphin gene was related to both the activation of nuclear embedded isozymes of protein kinase C (PKC) and cytosolic Ca\textsuperscript{2+} overload,\textsuperscript{16,17} suggesting the recruitment of crucial signaling pathways. Interestingly, in cardiomyopathic myocytes, secreted dynorphin B elicited a tonic feed-forward stimulation of its coding gene.\textsuperscript{18} Moreover, recently uncovered nuclear opioid receptors were found to be coupled to the activation of both nuclear PKC activity and prodynorphin gene transcription.\textsuperscript{19} These findings suggest that an opioid gene may play an autocrine and intracrine role in the regulation of myocardial homeostasis.

In the present study, we used P19 ES cells to examine prodynorphin gene transcription and dynorphin B expression throughout different stages of DMSO-induced cardiac differentiation. In this in vitro model, we also aimed to elucidate whether the expression of a cardiogenic program may be triggered by the exposure of P19 cells to dynorphin B in the absence of DMSO. A potential role of the prodynorphin gene and endogenous dynorphin B in cardiogenesis was explored.

Materials and Methods

Cell Differentiation

P19 cells (European Collection of Cell Cultures) were grown and differentiated as previously described.\textsuperscript{3} Briefly, \textsuperscript{10\(^5\)} cells/mL were grown in suspension in 60-mm bacteriological Petri dishes (Falcon) in medium containing 1% DMSO or the indicated concentrations of dynorphin B. At day 2, the medium was changed to fresh DMSO-containing medium, and at day 4 the aggregates were plated into tissue culture-grade dishes (Falcon) and maintained without any drug. Days of differentiation were numbered consecutively after the first day of aggregation.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted at the indicated time and reverse transcribed. The resulting cDNA was then amplified according to the reaction conditions previously reported.\textsuperscript{20} Using 35 PCR cycles, the products of the investigated genes were all within the linear phase of the reaction. Specific primers for MHC, myosin light chain-2V (MLC), GATA-4, Nkx-2.5, and GAPDH are described elsewhere (see expanded Materials and Methods online, available at http://www.circresaha.org). Specific primer pairs against mouse prodynorphin gene and dynorphin B were designed elsewhere.\textsuperscript{3,21} According to previous studies,\textsuperscript{8,9} basal mRNA expression of the prodynorphin gene was 5\textsuperscript{\textast}–CTGCTGACCGGATGAATGAA–3\textsuperscript{\textast} and 5\textsuperscript{\textast}–GCAGCGGAACAAGCACAGAGGAT–3\textsuperscript{\textast}. RT-PCR conditions were 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minute.

Analysis of Prodynorphin Gene Expression

Prodynorphin mRNA was also assessed by solution hybridization RNase protection.\textsuperscript{13,22} Briefly, a 424-bp fragment of the main exon of mouse genomic prodynorphin clone was inserted into pCRII-TOPO (Invitrogen). Transcription of the plasmid linearized with Apal generated a sense strand of prodynorphin mRNA used to construct a standard curve of prodynorphin mRNA, whereas transcription of the plasmid linearized with BamHI in the presence of [\textsuperscript{\textast}P]CTP (800 Ci/mmol) gave an antisense strand used to hybridize cellular prodynorphin mRNA.

Nuclear runoff transcription assay was performed in isolated nuclei, as described.\textsuperscript{16} Nuclear RNA was subjected to RNase protection assay. \textsuperscript{32}P-labeled RNA was hybridized for 12 hours at 55°C with unlabeled antisense prodynorphin mRNA synthesized, as described above. \textsuperscript{32}P-labeled nuclear RNA was also hybridized with unlabeled antisense cyclophilin mRNA, used as a control for control. Immunoreactive dynorphin B was measured by a previously described radioimmunoassay procedure.\textsuperscript{10,16}

Transfections

Undifferentiated P19 cells were transfected with 200 nmol/L prodynorphin antisense phosphorothioate oligonucleotide or 200 nmol/L scrambled phosphorothioate oligomer (negative control) (Sequitur Inc) with the aid of cationic lipids (Oligofectins) provided by the manufacturer. Transfection was carried out for 4 hours at 37°C in a serum-free medium. Cells were then replaced in a serum-containing medium and, after a 24-hour period, were exposed to 1% DMSO. The transfection procedure was repeated at 48-hour intervals. A fluorescein-labeled derivative of the antisense oligonucleotide was used to determine transfection efficiency. Fluorescence microscopy performed at defined times after transfection revealed that \textasciitilde;90% of identifiable nuclei had taken up the fluorescein oligonucleotide and that nuclear staining persisted for the duration of the experiment (not shown). After 4 days of DMSO treatment, cells were fixed with 4% paraformaldehyde and MHC was identified by the MF 20 mouse antmyosin monoclonal antibody.\textsuperscript{21}

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Nkx-2.5 and GATA-4 gene expression was induced after 2 or 4 days of treatment with 1% DMSO, respectively (Figure 1). According to previous studies,\textsuperscript{8,9} basal mRNA expression was not detectable in P19 cells cultured in the absence of the differentiator. DMSO-mediated induction of GATA-4 and Nkx-2.5 preceded the expression of MHC and MLC genes, two cardiac muscle genes (Figure 1).

In this study, we examined whether the increase in GATA-4 and Nkx-2.5 gene expression elicited by DMSO may be associated with the expression of an endogenous growth regulator in the early phase of the cardiac commitment of P19 cells. Figure 2 shows that the prodynorphin gene was expressed under basal conditions in cells cultured without DMSO. RT-PCR suggested a substantial increase in prodynorphin mRNA after cell exposure to DMSO (Figure 2A). Such an effect was additionally confirmed by RNase...
protection experiments revealing that prodynorphin gene expression was already enhanced after 2 days in the presence of DMSO and reached a maximum after 4 days of treatment. In vitro runoff assays performed in nuclei isolated from P19 cells indicated that the DMSO effect occurred at the transcriptional level (Figure 2C).

Consistent amounts of dynorphin B were detected in P19 cells and their incubation media (Figure 2D). Confirming our previous observations in myocardial cells, 10,16 the intracellular levels of dynorphin B (ir-dyn B), Gray bars indicate ir-dyn B in cells; and white bars, ir-dyn B in the medium. Values are mean±SE (n=6). §Value of white bar is significantly different from that of the gray bar; *Significantly different from untreated controls (–). An expanded version of the legend for Figure 2 is available online at http://www.circresaha.org.

The finding that DMSO-triggered induction of cardiogenic genes was preceded by the activation of prodynorphin gene and dynorphin B expression suggests that an opioid gene may be a suitable candidate for the initiation of a cardiac program of differentiation. In the absence of DMSO, 1 μmol/L dynorphin B was able to induce the expression of Nkx-2.5 and GATA-4 genes after 1 or 2 days of treatment, respectively (Figure 3). Exposure to dynorphin B also enhanced MHC and MLC gene expression (Figure 3). The dose-response curve of the effect of dynorphin B on Nkx-2.5 and GATA-4 mRNA expression revealed that the opioid peptide was effective at a concentration as low as 10^-5 mol/L (Figure 3). Figure 3 shows that the opioid effect was specific in nature, because it was abolished by Mr-1452, a selective κ-opioid receptor antagonist. 19 MHC expression was additionally assessed in immunofluorescence studies with the aid of the mouse MF 20 monoclonal antibody. Whereas MF 20–positive cells were not detectable in the absence of both DMSO and dynorphin B (Figure 4A), MF 20 labeling was clearly observed in DMSO-treated cells (Figure 4B). Positive staining was also observed in P19 cells exposed to dynorphin B in the absence of DMSO (Figure 4C). The pattern of MF 20 staining in dynorphin B–treated cells was superimposable to that observed in cells exposed to DMSO, providing additional evidence for the involvement of dynorphin B in the activation of a cardiogenic program.

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**Figure 2.** DMSO triggers prodynorphin gene and dynorphin B expression in P19 cells. A, RT-PCR analysis of prodynorphin (prodyn) mRNA. B, RNase protection analysis of prodynorphin mRNA expression. Upper part of the panel shows representative autoradiograms of prodynorphin mRNA. Averaged values of prodynorphin mRNA expression (mean±SE; n=6) are reported in the lower part of the panel. *Significantly different from untreated controls (–). C, Nuclear runoff assay. a, Transcription of the prodynorphin gene. b, Cyclophilin mRNA. D, Immunoreactive dynorphin B (ir-dyn B). Gray bars indicate ir-dyn B in cells; and white bars, ir-dyn B in the medium. Values are mean±SE (n=6). §Value of white bar is significantly different from that of the gray bar; *Significantly different from untreated controls (–). An expanded version of the legend for Figure 2 is available online at http://www.circresaha.org.

**Figure 3.** Effect of dynorphin B on the expression of a distinct cardiogenic program. P19 cells cultured without DMSO were treated in the absence or presence of dynorphin B. Total RNA was extracted and processed for RT-PCR analysis of the indicated transcripts. Ethidium bromide–stained gels represent the time course (top) or the dose response (bottom) of the effect of dynorphin B on targeted gene expression. Mr indicates cells cultured in the presence of 1 μmol/L dynorphin B and 1 μmol/L Mr-1452.

**Figure 4.** Dynorphin B induces cardiomyogenesis in P19 cells. P19 cells were aggregated for 4 days in the absence of both DMSO and dynorphin B (A), in the presence of 1% DMSO (B), or in the presence of 1 μmol/L dynorphin B (C) and stained on day 5 with MF 20, an anti-MHC monoclonal antibody. Bar=40 μm.
The cardiogenic effect of an exogenously administered opioid agonist prompted us to investigate whether an intrinsically produced opioid peptide may elicit cardiogenesis through an autocrine mechanism. For this purpose, cell aggregation with DMSO, a treatment enhancing the synthesis and secretion of dynorphin B, was performed in the presence of an opioid receptor antagonist. Figure 5 shows that Mr-1452 downregulated DMSO-induced GATA-4 and Nkx-2.5 mRNA expression as well as DMSO-elicited activation of MHC and MLC genes. Specificity of the antagonistic effect of Mr-1452 was inferred by the finding that the inactive enantiomer Mr-1453 failed to abolish the DMSO action (Figure 5). We next assessed MHC expression in P19 cells transfected with a prodynorphin antisense phosphorothioate oligonucleotide. In cells primed for 4 days with 1% DMSO, transfection resulted in an 80% to 90% decrease in both intracellular and secreted dynorphin B compared with nontransfected DMSO-treated cells (Figure 6). The immunofluorescence analysis of MF 20 staining revealed that P19 prodynorphin antisense transfectants failed to develop in MHC-positive cells in response to DMSO (Figure 7). The treatment of P19 cells with Mr-1452 was also able to prevent the appearance of MF 20 staining in DMSO-exposed cells (Figure 7).

Discussion

Concerted activation of regulatory genes plays a fundamental role in determining the temporal and spatial patterns of embryonic development. Lineage-restricted transcription factors that regulate tissue-specific genes are especially important for tissue differentiation. Although the cardiac homeobox gene Csx/Nkx-2.5 and zinc finger–containing GATA-4 have been found to be essential for normal heart development, little is known about the molecular mechanism underlying their activation. Pluripotent P19 cells provide the opportunity of identifying intrinsic primers that may mimic the capability of DMSO to trigger the expression of genes involved in cardiogenic decision. The possibility that the activation of an endorphinergic system may act as an endogenous primer in the induction of cardiogenic genes is supported by the present observation that P19 cells express the prodynorphin gene and that DMSO-primed Nkx-2.5 and GATA-4 gene expression was preceded by a marked increase in expression of both prodynorphin gene and dynorphin B. Consistent with the role of the prodynorphin gene in specifying the cardiac lineage is the finding that the exposure of P19 cells to dynorphin B...
triggers the expression of both Nkx-2.5 and GATA-4 as well as the activation of cardiac-specific genes. A cardiogenic role of the prodynorphin gene is also supported by the finding that dynorphin B–treated cells were positively stained with MF 20, a specific anti-MHC antibody. In the present study, MF 20 staining was clearly evident after treatment for 4 days in the presence of DMSO or dynorphin B. This observation excludes the possibility that skeletal muscle cells may contribute to MHC expression, because in P19 cells, skeletal muscle does not appear until after 9 to 10 days of aggregation with DMSO.\textsuperscript{7,8} Additional evidence correlating the immuno-fluorescence results to the onset of a cardiac phenotype is provided by the observation that MF 20 staining was associated with the appearance of MLC transcript, a cardiac marker for ventricular chamber specification.\textsuperscript{6} The present finding that dynorphin B was synthesized and secreted from DMSO-treated P19 cells and that a specific \( \kappa \)-opioid receptor antagonist suppressed the DMSO effect on cardiacogenic and cardiac-specific genes prompts the hypothesis that cardiogenesis may be controlled by an opioid–related autocrine environment. Such a hypothesis is substantiated by the observation that both antisense-mediated downregulation of the prodynorphin gene and opioid receptor antagonism counteracted MHC expression.

Previous studies\textsuperscript{22,23} have established that both Nkx-2.5 and GATA-4 can be induced in mesoderm-originated precardiac cells by endoderm-derived signals comprising several members of bone morphogenetic proteins (BMPs) belonging to the transforming growth factor-\( \beta \) superfamily. It is now evident that both BMP receptors and DMSO convey differentiating stimuli to the nuclear transcriptional machinery through common signal transduction pathways that involve the activation of mitogen-activated protein kinases (MAPKs).\textsuperscript{24,25} DMSO also elicits intracellular Ca\textsuperscript{2+} overload and phosphoinositide turnover, thereby increasing the cellular availability of diacylglycerol and the activity of PKC isozymes.\textsuperscript{26,27} Interestingly, opioid receptor stimulation is coupled to the activation of both PKC-16,28,29 and MAPK-mediated pathways\textsuperscript{30} and has also been shown to affect phosphoinositide turnover,\textsuperscript{12,31} promoting the release of Ca\textsuperscript{2+} from an intracellular storage site.\textsuperscript{10–12,18} In addition, an upstream initiator-like sequence in the promoter of the prodynorphin gene has been shown to be recognized by a DNA-binding protein acting as a tyrosine kinase substrate.\textsuperscript{32} Hence, opioid-related signaling shares crucial developmental pathways with both BMP- and DMSO-activated cascades. Opioid peptides have also been shown to be nuclear proteins responsive to growth arrest and differentiation signals.\textsuperscript{33} In this regard, the opioid peptide precursor preprodynorphin has been found to display structure similarity with the helix-loop-helix motif of multiple DNA-binding proteins and to exhibit cysteine-rich regions characteristic of zinc-finger domains.\textsuperscript{34} Moreover, dynorphin B proved to be a specific ligand of nuclear opioid receptors in myocardial cells.\textsuperscript{19} These receptors were overexpressed in nuclei of myocytes isolated from Syrian cardiomyopathic hamsters and coupled to nuclear PKC activation and prodynorphin gene transcription, suggesting that intracrine signals for growth regulation may be fashioned by an opioid gene.

The possibility that the prodynorphin gene may be involved in the orchestration of cardiogenesis may be of particular biological relevance. The attempt at rescuing the cardiac function in damaged myocardium is now an area of inquiry.\textsuperscript{35} Damaged skeletal muscle is able to regenerate because of the presence of satellite cells, which are undifferentiated myoblasts.\textsuperscript{36} In contrast, destruction of cardiac myocytes is associated with an irreversible loss of myocardium and replacement with scar tissue, because it lacks stem cells.\textsuperscript{36} Growing evidence indicates that stem cells obtained from skeletal muscle can be implanted into injured myocardium and are able to differentiate into cardiac muscle fibers,\textsuperscript{37} thereby providing a unique opportunity to repair damaged heart muscle. Strikingly, besides showing clear morphological evidence of a cardiac phenotype, the implanted cells exhibited long-term survival within the context of recipient myocardium.\textsuperscript{38} These findings are consistent with the hypothesis of milieu-influenced differentiation of the implanted stem cells into cardiogenic-like muscle cells. In previous investigations, we have shown that as a result of prodynorphin gene expression, dynorphin B is both synthesized and secreted from adult myocytes.\textsuperscript{10,16–18} In this study, we show that this opioid peptide is also secreted by an ES cell and may act in an autocrine fashion to elicit the appearance of a cardiac phenotype. Therefore, cardiac myocytes within the recipient myocardium and implanted stem cells might both generate a cardiogenic program involving the expression of an opioid gene and that of its related peptide product. In addition, dynorphin B itself has previously been shown to enhance prodynorphin gene expression in adult myocytes.\textsuperscript{18,19} suggesting that the local availability of dynorphin B may be enhanced by a paracrine crosstalk among myocytes and transplanted stem cells.

It is becoming evident that ES cells can be differentiated in vitro and used as a novel source of cells for transplantation.\textsuperscript{38} The results of the present study provide evidence that formation of a myocardial cell can be triggered in vitro by an opioid gene. Additional studies are needed to verify whether manipulation of the prodynorphin gene may prove effective in triggering cardiac differentiation in human stem cells implanted within an in vivo environment. Clarification of this issue must await more direct functional studies and may pave the way for developmental approaches to tissue engineering and myocardial regeneration.

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### References

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3. Biben C, Harvey RP. Homeodomains factor Nkx-2.5 controls left/right asymmetric expression of HHHL gene eHand during murine heart develop-


13. Ladd AN, Yatskievych TA, Antin PB. Regulation of avian cardiac myo-


19. Ventura C, Maioli M, Pintus G, Posadino AM, Tadolini B. Nuclear opioid receptors activate opioid peptide gene transcription in isolated myocardi-

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Supplementary: Expanded Materials and Methods

Opioid Peptide Gene Expression Primes Cardiogenesis in Embryonal Pluripotent Stem Cells

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Materials and Methods

α-minimum essential medium, calf serum, foetal bovine serum, M-MLV reverse transcriptase, Taq polymerase, DNA molecular weight markers and dNTP were purchased from Gibco. Restriction enzymes were from Boehringer Mannheim. The pBS vector was from Promega. \([\alpha^{-32P}]dCTP\), \([\beta^{-32P}]dCTP\) and \([\alpha^{-32P}]UTP\) were from Amersham International. RNAMATRIX™ was from BIO 101, Inc. (Vista, CA). Dynorphin B was purchased from Neosystem Laboratoire (Strasbourg, France). Certified peptide purity was 98% and was confirmed by reverse-phase high performance liquid chromatography. Dynorphin B was received as a lyophilized water-soluble peptide and was dissolved in the culture medium immediately before use. All the other chemicals were from Sigma.

RNA extraction and RT-PCR

Total RNA was extracted at the indicated time points according to the procedure described by Chomczynski and Sacchi (1). Total RNA (1μg) was reverse-transcribed for 45 min at 37 °C. The reaction was performed in a solution of 25 μl containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 0.2 mM of each dNTP, 0.1 μg of oligo(dT), and 200 U of M-MLV reverse transcriptase. The reaction mixture was then heated at 95 °C for 5 min to inactivate the enzyme. PCR amplification was performed in 25 μl of a reaction mixture containing 5 μl of the reverse-transcribed cDNA, 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 2.5 U of Taq polymerase, 0.2 mM of each dNTP and 50 pmol of sense and antisense primers that were previously dissolved in TE solutions (10 mM Tris, pH 8, 1 mM EDTA, pH 8.0). The number of amplification cycles was determined experimentally for each primer pair by establishing the point at which exponential accumulation plateaus. Aliquots of the PCR reactions, including 1 μCi of \([\alpha^{-32P}]dCTP\) (3000 Ci/mmol) were taken after 10, 15, 20, 25, 30, 35 and 40 cycles. Then, \([\alpha^{-32P}]dCTP\)-labelled PCR products were electrophoresed on 2% agarose gels. Ethidium bromide-stained bands were excised under UV light and the radioactivity incorporated into PCR products was determined.
by β-scintillation counting. Under these experimental conditions and using 35 PCR cycles, the products of the investigated genes were all within the linear phase of the reaction. The position of PCR fragments was evaluated by comparison with a DNA molecular weight marker. GAPDH mRNA was used for each sample as an internal control for mRNA integrity and equal loading. The levels of radioactivity incorporated into each targeted product were normalized by comparison with the levels of radioactivity incorporated into the GAPDH product from the same sample. The oligonucleotide sequences and PCR conditions for the detection of α-myosin heavy chain (MHC), myosin light chain-2V (MLC), GATA-4, Nkx-2.5 and GAPDH were previously described (2-4). Specific primer pairs directed against mouse prodynorphin gene were: 5’-CTGCTGACCGGATGAATGATGAA-3’ and 5’-GCAGCGGAAACAAGCACAAGGGAT-3’. PCR conditions were 94 °C for 30 sec, 59 °C for 30 sec and 72 °C for 1 min.

**Nuclear Run-off Transcription Assay**

The nuclear preparation lacked contamination by sarcoplasmic reticular membranes, inner or outer mitochondrial membranes or sarcolemmal membranes, as indicated by the measurement of the activity of the corresponding marker enzymes rotenone-insensitive NADPH cytochrome c reductase, succinate dehydrogenase, rotenone-insensitive NADH cytochrome c reductase, 5’ nucleotidase and Na’\(\text{,K}^{+}\)-ATPase, which were all undetectable in the nuclear fraction (not shown). Nuclei were resuspended in a buffer containing 50 mM Tris/HCl, pH 8.0, 5 mM MgCl\(_2\), 0.1 mM EDTA, 40\% glycerol, 0.1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 10 mM β-mercaptoethanol. 90 μl of the nuclear preparation were added with 100 μl of 2 X reaction buffer containing 10 mM Tris/HCl, pH 7.5, 5 mM MgCl\(_2\), 0.3 M KCl, 5 mM DTT, 1 mM each of ATP, GTP, and CTP, and 5 μl of [α-\(^{32}\)P]UTP (3000 Ci/mmol), followed by incubation at room temperature for 15 min. DNA was digested by incubating the transcription mixture for 5 min at room temperature in the presence of 1 μl of 20,000 units/ml RNase-free DNase. Nuclear RNA was purified on RNAMATRIX\textsuperscript{TM} and then subjected to RNase protection assay. \(^{32}\)P-labeled RNA
(about $5 \times 10^6$ cpm) was hybridized for 12 h at 55 °C with unlabeled antisense prodynorphin mRNA. Samples were incubated with a combination of RNase A and T1 and exposed to proteinase K. The protected fragments were electrophoretically separated in a polyacrylamide nondenaturing gel. Autoradiographic exposure was for 48 h. $^{32}$P-labeled nuclear RNA was also hybridized with unlabeled antisense cyclophilin mRNA synthesized from a Neol-linearized pBS vector containing a 270-base pair fragment of plB15, a cDNA clone encoding for rat cyclophilin (5). Cyclophilin mRNA was utilized as a constant mRNA for control.

**Dynorphin B-like material**

Immunoreactive dynorphin B (ir-dyn B) was assessed by a radioimmunoassay method that utilized the 13 S antiserum raised against dynorphin B and capable of recognizing the high molecular weight peptides cleaved from the prodynorphin precursor and containing dynorphin B in their sequence (6). Acetic acid extracts from P19 cells cultured in the absence or presence of DMSO or dynorphin B, or pooled samples from their incubation medium were processed by reverse-phase high performance liquid chromatography. The collected fractions were radioimmunoassayed and the immunoreactivity was attributed to authentic dynorphin B by comparison with the elution position of a synthetic standard.

**Data Analysis**

The statistical analysis of the data was performed by using a one-way analysis of variance followed by Newman Keul’s test and assuming a $p$ value less than 0.05 as the limit of significance.

**References**


**Figure Legend**

**Figure 1 Online** (Same as Figure 2 in print version). DMSO triggers prodynorphin gene and dynorphin B expression in P19 cells. *(A)* ethidium bromide-stained gels of the RT-PCR analysis of prodynorphin (prodyn) mRNA. *(B)* RNase protection analysis of prodynorphin mRNA expression. The upper part of the panel shows representative autoradiograms relating to prodynorphin mRNA. Autoradiographic exposure was for 2 days on Kodak X-Omat film with an intensifying screen. The bar indicates the position of a 400-base pair radiolabeled DNA marker, showing that the single protected fragment migrated with a molecular size comparable to prodynorphin mRNA (424 bases). *(C)* Analysis of the rate of transcription of the prodynorphin gene in isolated nuclei. Nuclear run-off assay was performed as described under “Methods”. Autoradiographic exposure was as described in *(B). Row a*, transcription of the prodynorphin gene. *Row b*, cyclophilin mRNA. On the right are indicated the position of 400- or 220-base pair radiolabeled DNA markers, showing that the single protected fragments migrated with a molecular size comparable to prodynorphin mRNA (424
bases) or cyclophilin mRNA (270 bases). (D) Expression of immunoreactive dynorphin B (ir-dyn B). Each single value in the medium (white bar) was calculated in a final volume corresponding to the incubation medium from $10^6$ cells. Each experiment was performed in the presence of a peptidase inhibitor mixture containing 20 $\mu$M bestatin, 1 mM leucyl-L-leucine, 3 $\mu$M poly-L-lysine, 0.3 $\mu$M thiorphan, 30 $\mu$M 1-10-phenantroline, 6 $\mu$M dithiothreitol.