BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 66–70 (1997)
ARTICLE NO. RC976905

Identification of Heparin-Binding Sites in Midkine and Their Role in Neurite-Promotion

Toshinobu Asai,*,† Kiichi Watanabe,* Keiko Ichihara-Tanaka,* Norio Kaneda,*,‡ Soichi Kojima,§ Akihisa Iguchi,† Fuyuhiko Inagaki,¶ and Takashi Muramatsu*,[1]

**Department of Biochemistry and †Department of Geriatrics, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan; ‡Department of Biological Function and Analysis, Faculty of Pharmacy, Meijyo University, Nagoya 468, Japan; §The Laboratory of Gene Technology and Safety, The Institute of Physical and Chemical Research (RIKEN), Koyadai, Tsukuba, Ibaraki 305, Japan; and ¶Department of Molecular Physiology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan*

Received May 26, 1997

Midkine (MK) is a heparin-binding growth factor, which promotes neurite outgrowth in embryonic neurons and enhances their survival. The three dimensional structure of MK clarified by NMR spectroscopy indicates that several basic amino acids are exposed on the surface of the C-terminal half domain, which retains heparin-binding and neurite-promoting activity. We performed site-directed mutagenesis of these amino acids, and found that mutation of arginine^{78} reduced the heparin-binding activity. Mutation of either lysine^{83} or lysine^{84} scarcely affected heparin-binding activity, while the double mutant involving both lysine residues showed reduction in the activity. Neurite-promoting activity of mutant MKs always correlated with their heparin-binding activity, illustrating the close relationship of the two activities. Thus, the present result verifies the occurrence of two distinct heparin-binding sites involved in neurite-promoting activity of MK molecule.

Recognition of heparin and related carbohydrate structures by proteins is of fundamental importance in the regulation of cellular activities and body homeostasis (1,2). The classical example of this is recognition of heparin by anti-thrombin III, leading to inhibition of blood coagulation (1,3). Recently, several growth factors have been shown to interact with heparin-like domains in heparan sulfate proteoglycans present on the cell surface (2,4). Notably, the interaction of fibroblast growth factors (FGFs) with the cell-surface carbohydrate structure is essential for their biological activity: as a result of the interaction, FGFs form dimers, which is the key requirement for receptor dimerization, and thereby signaling (4,5).

Midkine (MK) (6,7) and heparin binding growth-associated molecule (HB-GAM) (8,9), also known as pleiotrophin (PTN) (10), form a new family of heparin-binding growth factors (11). They are structurally unrelated to FGFs and are 45% identical to each other (6–11). These molecules share many biological activities both *in vivo* and *in vitro*, whereas their modes of expression during development are quite different (12). Both promote neurite outgrowth of embryonic neurons (8,13), guide the neurites (14,15), transform NIH 3T3 cells to form malignant tumors (16,17) and enhance the fibrinolytic activity of aortic endothelial cells by elevating plasminogen activator (PA) activity (18,19). The neurite-promoting and neurite-guiding activities of MK and HB-GAM appear to be physiologically important as these molecules are present along the path of neuronal cell migration (14,20).

MK and HB-GAM are largely composed of two domains (21). The C-terminal half of the MK molecule (C-1/2 domain) has the conformation-dependent heparin-binding site and retains both neurite outgrowth-promoting activity (22) and PA-enhancing activity (23). This finding suggested that there may be a correlation between heparin-binding activity and biological activities of these molecules. Indeed, *N*-syndecan, a heparan sulfate proteoglycan, has been proposed to be a receptor of HB-GAM upon HB-GAM-induced neurite-outgrowth of embryonic neurons (24).

Recently, we determined the three dimensional structure of the MK molecule (25). Several basic amino acids which include a consensus of heparin binding sequence (1) are exposed in one side of the C-1/2 domain,

[1] Corresponding author. Fax: +81-52-744-2065. E-mail: tmurama@tsuru.med.nagoya-u.ac.jp.

Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco-modified minimum essential medium; FGF, fibroblast growth factor; HB-GAM, heparin binding-growth associated molecule; MK, midkine; PA, plasminogen activator; PTN, pleiotrophin.

0006-291X/97 $25.00

suggesting that some of these basic amino acids are involved in recognition of heparin-like structures. Since so far nothing is known about the actual heparin-binding site in MK or HB-GAM, we performed site-directed mutagenesis of these amino acids and examined the effect of these mutations on heparin-binding activity of MK. We also examined the effect of reduced heparin-binding activity of the mutated MK on the neurite-promoting activity.

MATERIALS AND METHODS

Construction of transfer vectors carrying various MK mutants. Site-directed mutagenesis was performed on a wild-type mouse MK cDNA subcloned into pBluescript M13 by the method of Ito et al. (26). The primers used were as follows: primer I (sense), $^{5'}$AGCTCG-GAATTAACCCTCACTAAAG$^{3'}$; primer II (antisense), $^{5'}$CACTGC-AGTCTGGCCTCCTGACTTAGTC$^{3'}$; primer III (sense), $^{5'}$GGCGAT-CCTATAAAT-ATGCAGCACCGAGGCTTCT$^{3'}$; primer IV (antisense), for MK(R78Q), $^{5'}$AGGGTCCCTTG-CTGGGCTTTGGTGCC$^{3'}$, for MK(K83Q), $^{5'}$ACCGCGCCTTCTGCAGGGTCCCTTGG$^{3'}$, for MK(K84Q), $^{5'}$TGTACCGCGCCTGCTTCAGGGTCCC$^{3'}$, and for MK(KK83,84QQ), $^{5'}$GTA-CCGCGCCTGCTGCAGGGTCCCTTGG$^{3'}$.

Introduction of mutations was confirmed by DNA sequencing using an automated fluorescence DNA sequencer (model 373A, Applied Biosystems, CA). cDNAs of MK(R78Q), MK(K83Q), MK(K84Q) mutants were subcloned into *Bam* HI-*PSt* I sites downstream of the *Autograph california* nuclear polyhedron promoter in the transfer vector pVL 1393 (Pharmingen, CA) as in the case of the wild-type cDNA (15). For MK(KK83,84QQ) mutant, the MK cDNA, from which the sequence encoding the signal sequence was removed by PCR, was subcloned into pBlueBacHis (B) (Invitrogen, CA) digested with *Bam* HI-*Nco* I and was blunt-ended by Klenow fragment to allow histidine tagging and to add an enterokinase-susceptible sequence. Then, the coding sequence obtained by PCR was digested with *Sma* I and *Bgl* II to modify both ends and the resultant fragment was subcloned into pMbac (Stratagene, CA) cleaved with *Sma* I and *Bam* HI to add the Melitin signal peptide. Thus, in MK(KK83,84QQ) mutant, the transfer vector had the Meliten signal peptide, histidine tag, enterokinase sequence and MK coding sequence. Similarly, a transfer vector with wild-type MK cDNA with a histidine tag was also constructed.

Purification of wild-type and mutant MK. The culture medium was collected and centrifuged at 7,000 rpm for 10 min, followed by ultracentrifugation at 35,000 rpm (100,000×g) for 30 min to remove virus particles. The supernatant (500 ml) thus obtained was directly applied to a Hi-Trap heparin column (column size, 1ml; Pharamacia Biotech., Uppsala), pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.8, containing 0.2 M NaCl. After washing the column with the same buffer, proteins were eluted with a linear NaCl gradient (0.2 to 2 M NaCl in 80 ml of 50 mM sodium phosphate buffer, pH 6.8). All column operations were performed using an FPLC system (Pharmacia Biotech, Uppsala) at a flow rate of 0.5 ml/min at 4°C.

Assay for neurite outgrowth on MK tracks. Single cells were isolated from rat embryonic cerebral cortex (embryonic day 17–18) as described by Kaneda et al. (15). The formation of a pattern of MK on plastic culture plates was performed according to the method of Rauvala et al. (14). Briefly, culture plates (24 wells, Falcon 3057; Becton Dickinson, NJ) were incubated with purified MK or the various mutants at a concentration of 10 μg/ml for 2 h at room temperature. After washing twice with H_2O, the plates were dried, and metal electron microscopy grids (HDL 200; Veco, Amsterdam) were placed in the wells, followed by irradiation with UV light at 315 nm for 30 min in a UV chamber (GS Gene Linker; Bio-Rad, CA) to produce a grid pattern of the substrate. After removing the grids, the wells were incubated with DMEM containing 1% BSA for 30 min at 37 °C, and washed twice with H_2O. Then, suspensions of the rat brain nerve cells were seeded at a density of 0.8×10^6 cells per well, and cultured for 48 h at 37°C under an atmosphere of 5% CO_2 and 95% air.

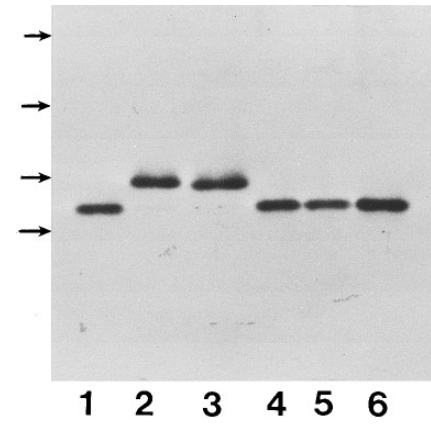

FIG. 1. SDS-polyacrylamide gel electrophoresis of mutant MKs purified by heparin agarose affinity chromatography. About 100 ng proteins were analyzed on a 12.5% running gels, and proteins were visualized by silver reagent. Arrows indicate the position of protein standards; from the top, they were ovalbumin (45 kDa), carbonic anhydrolase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lyzozyme (14.5 kDa). MK migrates as a 17 kDa band due to high degree of basicity (7). 1, wild-type MK; 2, His-MK; 3, His-MK (KK83, 84 QQ); 4, MK (R78Q); 5, MK (K83Q); 6, MK (K84Q).

Analytical procedures. SDS-polyacrylamide gel electrophoresis (12.5% gels) was carried out by the method of Laemmli (27). The gels were then stained with silver reagent, or subjected to Western blotting (28) using a specific anti-MK antiserum described previously (13). The protein concentration was measured by the micro BCA assay (Pierce, IL) using BSA as a standard.

RESULTS

Heparin Binding Activity of Mutant MKs

The three dimensional structure of human MK recently clarified by NMR spectroscopy revealed the existence of a surface cluster of basic amino acids located in one side of C1/2-domain, suggesting that these amino acids may be candidates for heparin binding sites (25). These residues are K^{76}, R^{78}, K^{83}, K^{84}, R^{86} and K^{99} (numbered according to mouse MK). Among these K^{76}, R^{78}, K^{83} and K^{99} are conserved both in MK and HB-GAM of all species examined to date (29). On the other hand, K^{84} is conserved only in MK of various species, but changed to R in HB-GAM (29). To determine whether these basic amino acids function as heparin binding sites, we prepared mutant derivatives in which one of these basic amino acids was changed to glutamine by site-directed mutagenesis. Three mutants, R^{78}→Q [MK(R78Q)], K^{83}→Q[MK(K83Q)], and K^{84}→Q [MK(K84Q)], were secreted and could be purified to homogeneity using heparin-agarose affinity chromatography (Fig. 1). When K^{76} or K^{99} were con-

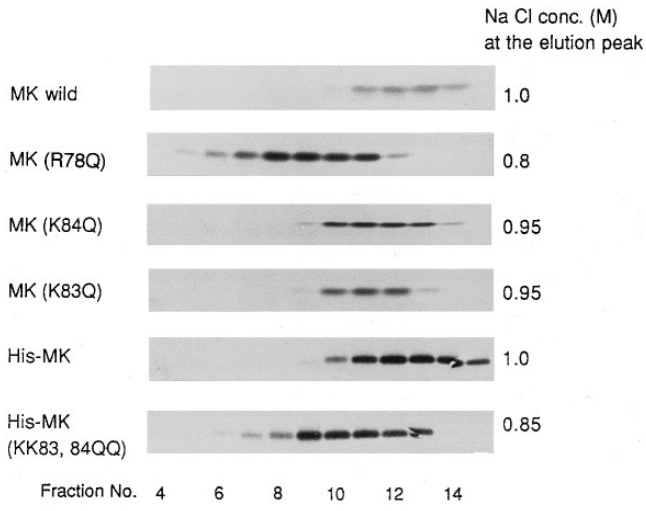

FIG. 2. Altered heparin binding activity of mutant MKs. Heparin agarose affinity chromatography was performed as described in MATERIALS AND METHODS and fractions, 2 ml were collected. The elution positions of wild-type and mutant MKs upon heparin-agarose affinity chromatography were determined by SDS-polyacrylamide gel electrophoresis on a 12.5% gels followed by Western blotting using anti-MK antibody. NaCl concentrations at which peaks of MK or its mutants were eluted are presented on the right side of the figure.

verted to Q, the mutant MKs were not secreted nor accumulated in the cells, suggesting that these amino acids were involved in stability of MK molecule.

We also produced MK (KK83, 84QQ) in which both K^{83} and K^{84} were changed to Q, to examine the effects of the double mutation. To facilitate its purification in case its heparin-affinity was greatly reduced, histidine oligomers were attached to the N-terminal side. However, the mutant protein termed His-MK (KK83, 84QQ) retained considerable heparin-binding activity, and could be purified to homogeneity by heparin-Sepharose column chromatography. We used the protein with a histidine tag, since attempts to remove the tag by enterokinase resulted in partial fragmentation of MK, probably due to the presence of large numbers of lysine residues. We also produced wild-type MK with histidine oligomers (His-MK).

We, at first, examined the heparin-binding activities of these mutants using heparin-agarose affinity chromatography. The elution positions of MK and the mutants from heparin-agarose affinity chromatography were monitored by SDS-polyacrylamide gel electrophoresis (Fig. 2). MK (R78Q) showed reduced heparin binding activity; wild-type MK was eluted at a NaCl concentration of 1.0 M, while MK (R78Q) was eluted at 0.8 M NaCl. MK (K83Q) and MK (K 84Q) showed only slightly reduced heparin binding activities: elution peaks were at a NaCl concentration of 0.95 M. In contrast, the double mutant, His-MK (KK83, 84QQ) showed reduced heparin binding activity relative to His-MK; the elution peak of the latter was at a NaCl concentration of 1.0 M, whereas that of the former was at 0.85 M NaCl. Therefore, R^{78}, K^{83} and K^{84} are all involved in heparin binding activity, and R^{78} is the most important at the single amino acid level.

Neurite-Promoting Activity of Mutant MKs

Tracks of MK were formed on the surfaces of culture dishes by MK coating and UV irradiation with an electron microscopy grid. When embryonic brain neurons were plated on these dishes, neurons attached and extend neurites along the path of wild-type MK (Fig. 3A) or His-MK (Fig. 3B). MK (K83Q) and MK(K84Q) showed neurite-promoting and guiding activity indistinguishable from wild-type MK (Figs. 3C and 3D). However, along the tracks of MK (R78Q) (Fig. 3E) or His-MK (KK83, 84QQ) (Fig. 3F), neurites were not orderly arranged along the tracks and large aggregates of cells were formed. Therefore, neurite-promoting activity of mutant MKs correlated with their heparin-binding activities.

DISCUSSION

Using the *in vitro* mutagenesis technique, we demonstrated that the three amino acids that are exposed on one side of the C1/2-domain of MK, namely, R^{78}, K^{83} and K^{84}, play important roles in heparin-binding activity of MK. Single mutation of R^{78} resulted in a decrease in heparin-binding, whereas single mutation of either K^{83} or K^{84} had little effect on heparin binding activity, although simultaneous mutation of both K^{83} and K^{84} significantly reduced the binding capability. There is evidence that arginine residues interact with heparin more strongly than lysine residues (30,31); for example a heptapeptide of arginine binds to a heparin-agarose column more strongly than that of lysine (30), consistent with the present observation that mutation of one arginine residue had a stronger influence on heparin-binding activity than mutation of one lysine residue.

The K^{83} and K^{84} residues in MK are present within a consensus heparin-binding sequence, XXBX (X=basic amino acids, B=other amino acids) (1), whereas R^{78}, which was shown to be the most important in heparin-binding at the single amino acid level, is not included in an evident heparin binding sequence. These results agree with the recent findings that heparin-binding sites cannot necessarily be defined by primary sequence, and three dimensional structure to form a cluster of basic amino acids is more important (32,33). In the case of anti-thrombin III, R^{14}, K^{40}, R^{47}, R^{48}, K^{126}, R^{130} and R^{133} have been implicated in heparin-binding (32). For binding of basic FGF to heparin, K^{119}, K^{125}, K^{129} and K^{135} are important (34,35,36). MK is the third case in which the heparin-binding site has been re-

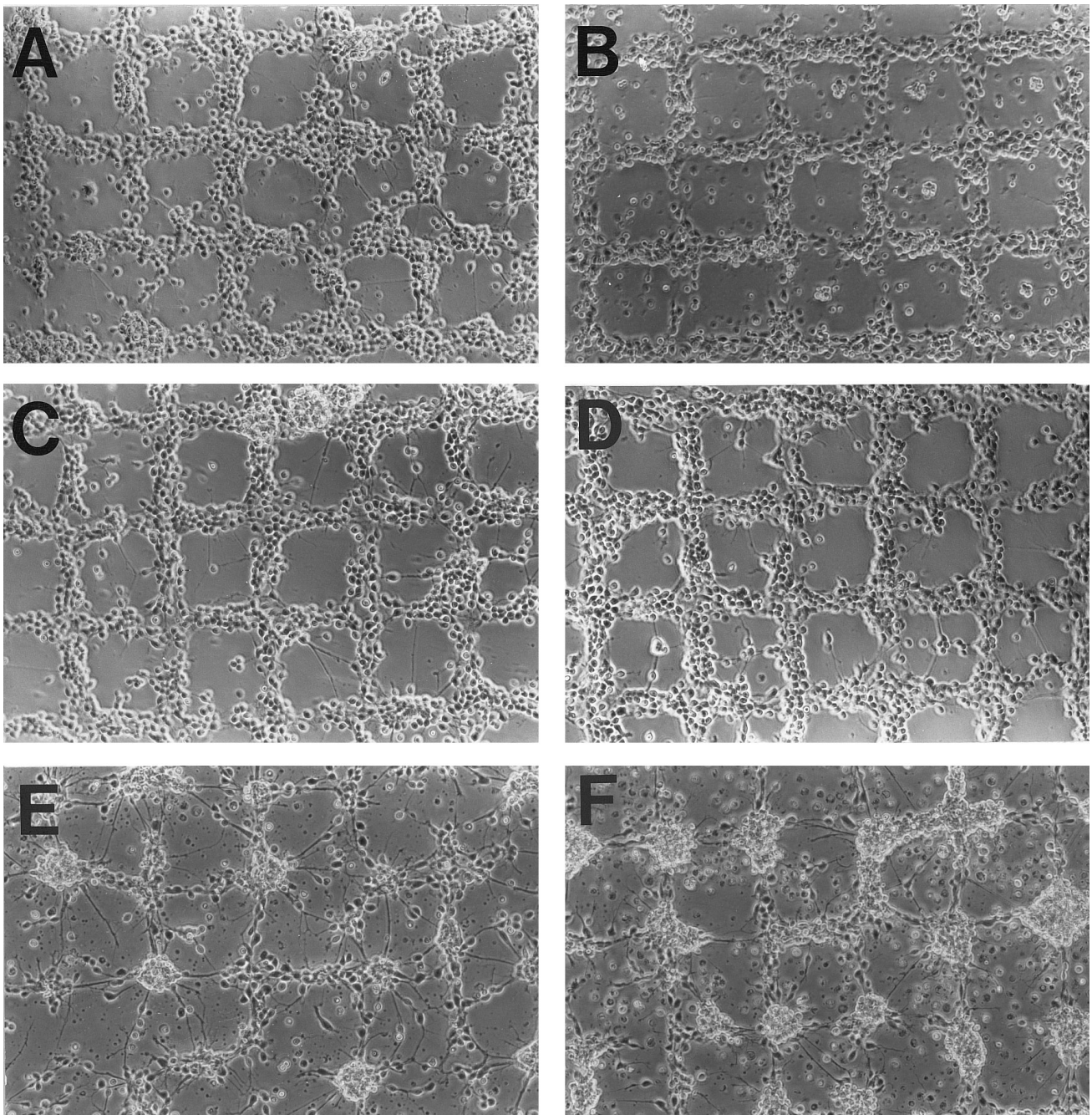

FIG. 3. Neurite outgrowth-promoting activity of mutant MKs as estimated by assay on patterned MK. Patterns of neurite outgrowth were recorded after culture for 48 h on grids of MK or its mutants by taking photomicrographs using an Olympus inverted phase contrast microscope. The pitch of grids was 125 μm. A, wild-type MK; B, His-MK; C, MK (K83Q); D, MK(K84Q); E, MK (R78Q), F, His-MK (KK83, 84QQ).

vealed in proteins with clarified three dimensional structure.

In the three dimensional structure of MK, basic amino acids exposed in one side of the C-terminal half of MK actually form two clusters, suggesting that there are two heparin-binding sites in it. R^{78} is in Cluster I, while K^{83} and K^{84} are in Cluster II (25). Thus, the present result verified that both Cluster I and II are actually involved in heparin-binding.

Finally, the mutants which had reduced heparin-binding activity showed reduced neurite-promoting activity. This observation establishes a close relationship between heparin-binding activity and neurite-promoting activity of MK.

ACKNOWLEDGMENTS

We thank Ms A. Horisawa and A. Miyata for their secretarial assistance. This work was supported by grants from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

1. Cardin, A. D., and Weintraub, H. J. R. (1989) *Arteriosclerosis* **9,** 21–32.
2. Ruoslahti, E., and Yamaguchi, Y. (1991) *Cell* **64,** 867–869.
3. Petitou, M., Duchaussoy, P., Lederman, I., Choay, J., Jacquinet, J. C., Sinaÿ, P., and Torri, G. (1987) *Carbohyd. Res.* **167,** 67–75.
4. Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell* **64,** 841–848.
5. Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I. (1994) *Cell* **79,** 1015–1024.
6. Kadomatsu, K., Tomomura, M., and Muramatsu, T. (1988) *Biochem. Biophys. Res. Commun.* **151,** 1312–1318.
7. Tomomura, M., Kadomatsu, K., Matsubara, S., and Muramatsu, T. (1990) *J. Biol. Chem.* **265,** 10765–10770.
8. Rauvala, H. (1989) *EMBO J.* **8,** 2933–2941.
9. Merenmies, J., and Rauvala, H. (1990) *J. Biol. Chem.* **265,** 16721–16724.
10. Li, Y.-S., Milner, P. G., Chauhan, A. K., Watson, M. A., Hoffman, R. M., Kodner, C. M., Milbrandt, J., and Deuel, T. F. (1990) *Science* **250,** 1690–1694.
11. Muramatsu, T. (1994) *Dev. Growth Differ.* **36,** 1–8.
12. Mitsiadis, T. A., Salmivirta, M., Muramatsu, T., Muramatsu, H., Rauvala, H., Lehtonen, E., Jalkanen, M., and Thesleff, I. (1995) *Development* **121,** 37–51.
13. Muramatsu, H., Shirahama, H., Yonezawa, S., Maruta, H., and Muramatsu, T. (1993) *Dev. Biol.* **159,** 392–402.
14. Rauvala, H., Vanhala, A., Castrén, E., Nolo, R., Raulo, E., Merenmies, J., and Panula, P. (1994) *Dev. Brain Res.* **79,** 157–176.
15. Kaneda, N., Talukder, A. H., Nishiyama, H., Koizumi, S., and Muramatsu, T. (1996) *J. Biochem.* **119,** 1150–1156.
16. Chauhan, A. K., Li, Y., and Deuel, T. F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 679–682.
17. Kadomatsu, K., Hagihara, M., Akhter, S., Fan, Q-W., Muramatsu, H., and Muramatsu, T. (1997) *Brit. J. Cancer* **75,** 354–359.
18. Kojima, S., Muramatsu, H., Amanuma, H., and Muramatsu, T. (1995) *J. Biol. Chem.* **270,** 9590–9596.
19. Kojima, S., Inui, T., Muramatsu, H., Kimura, T., Sakakibara, S., and Muramatsu, T. (1995) *Biochem. Biophys. Res. Commun.* **216,** 574–581.
20. Matsumoto, K., Wanaka, A., Takatsuji, K., Muramatsu, H., Muramatsu, T., and Tohyama, M. (1994) *Dev. Brain Res.* **79,** 229–241.
21. Fabri, L., Maruta, H., Muramatsu, H., Muramatsu, T., Simpson, R. J., Burgess, A. W., and Nice, E. C. (1993) *J. Chromatog.* **646,** 213–226.
22. Muramatsu, H., Inui, T., Kimura, T., Sakakibara, S., Song, X., Murata, H., and Muramatsu, T. (1994) *Biochem. Biophys. Res. Commun.* **203,** 1131–1139.
23. Kojima, S., Inui, T., Kimura, T., Sakakibara, S., Muramatsu, H., Amanuma, H., Maruta, H., and Muramatsu, T. (1995) *Biochem. Biophys. Res. Commun.* **206,** 468–473.
24. Raulo, E., Chernousov, M. A., Carey, D. J., Nolo, R., and Rauvala, H. (1994) *J. Biol. Chem.* **269,** 12999–13004.
25. Iwasaki, W., Nagata, K., Hatanaka, H., Inui, T., Kimura, T., Muramatsu, T., Yoshida, K., Tasumi, M., and Inagaki, F., submitted.
26. Ito, W., Ishiguro, H., and Kurosawa, Y. (1991) *Gene* **102,** 67–70.
27. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
28. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
29. Sekiguchi, K., Yokota, C., Asashima, M., Kaname, T., Fan, Q.-W., Muramatsu, T., and Kadomatsu, K. (1995) *J. Biochem.* **118,** 94–100.
30. Fromm, J. R., Hileman, R. E., Caldwell, E. E. O., Weiler, J. M., and Linhardt, R. J. (1995) *Arch. Biochem. Biophys.* **323,** 279–287.
31. Mascotti, D. P., and Lohman, T. M. (1995) *Biochemistry* **34,** 2908–2915.
32. Mourey, L., Samama, J.-P., Delarue, M., Petitou, M., Choay, J., and Moras, D. (1993) *J. Mol. Biol.* **232,** 223–241.
33. Busby, T. F., Argaves, W. S., Brew, S. A., Pechik, I., Gilliland, G. L., and Ingham, K. C. (1995) *J. Biol. Chem.* **270,** 18558–18562.
34. Li, L.-Y., Safran, M., Ariezer, D., Böhlen, P., Seddon, A. P., and Yayon, A. (1994) *Biochemistry* **33,** 10999–11007.
35. Zhang, J., Cousens, L. S., Barr, P. J., and Sprang, S. R. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3446–3450.
36. Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., and Rees, D. C. (1991) *Science* **251,** 90–93.