The mammalian achaete-scute homolog 1 (MASH1) protein is required for the early development of the nervous system. However, the molecular and biochemical mechanism by which MASH1 acts to determine neurogenesis are still unknown. The myocyte enhancer factor 2A (MEF2A) is a MADS transcription factor that is essential for the specification and differentiation of the muscle lineage. Here we show that MEF2A and MASH1 are coordinately induced during the differentiation of the teratocarcinoma cell line P19 along a neuronal lineage and that in transient transfection assays, MEF2A and MASH1 cooperatively activate gene expression. This cooperativity appears to be due to a specific physical interaction between MEF2A and MASH1. Taken together, these findings suggest that MASH1 via a cooperative interaction with MEF2A may regulate the expression of specific genes that are critical for neuronal differentiation.

We have recently reported that ectopic expression of MEF2<sup>1</sup> proteins in responsive cells induces and is required for the specification and differentiation of the myogenic pathway through the induction of the bHLH myogenic gene family (1). Moreover, activation of skeletal muscle genes requires cooperative interaction between MEF2 and the myogenic bHLH proteins. Thus, skeletal myogenesis is mediated by two distinct families of interactive transcription factors either of which can initiate the developmental cascade.

Interestingly, in addition to its expression in muscle cell types (2–4), the MEF2 genes are also expressed in the nervous system (5–7), but their role in neurogenesis has not been established. Based on their pattern of expression and the similarities between myogenesis and neurogenesis (8), we propose a general model wherein the MEF2 family participates in the myogenic and neurogenic pathways by cooperative interaction with cell type-specific transcriptional regulators in these cell lineages. These observations raised the possibility that the cell type restricted MEF2 transcription factors might be the partners of the neuronal specific bHLH regulator MASH1 in a manner similar to the interaction between MEF2 and MyoD in myogenesis. We show here that, although different from MyoD in primary structure, the neural specific bHLH protein MASH1 physically interacts with MEF2A and the two together cooperatively activate gene expression.

EXPERIMENTAL PROCEDURES

Cell Lines—Mouse teratocarcinoma cell line P19, COS, and the African green monkey kidney cell CV1 were obtained from American Type Cell Culture. P19 cells were maintained in Dulbecco’s modified Eagle’s medium with 7.5% calf serum and 2.5% fetal bovine serum and differentiated along a neuronal lineage by exposure to retinoic acid as previously described (9–11). CV1 and COS cells were cultured in 10% calf serum.

Antibodies—The anti-MASH1 monoclonal antibody was provided by Dr. David Anderson. The anti-MAP2 was gift from Dr. R. Vale. Rabbit anti-MEF2A antibody was generated in the laboratory as described (1). Goat rhodamine-conjugated anti-rabbit or anti-mouse IgG and fluorescein-conjugated anti-rabbit or anti-rabbit IgG were purchased from either Sigma or Boehringer Mannheim, respectively. Anti-GFAP was purchased from Sigma.

Indirect Immunofluorescence Assay—P19 cells were treated with retinoic acid and allowed to aggregates for 1 or 2 days. After culture for various days in the absence of retinoic acid, cells were either dispersed by trypsinization and cultured for 1 day, then fixed or fixed directly, and assayed by indirect immunofluorescence as described (5, 6).

Western Blot Analysis—P19 cells were differentiated as described above. Whole cell protein extracts were prepared as described elsewhere (5, 6) and Western blot analysis was performed with ECL (Amersham Corp.) reagents following the manufacturer’s instruction.

Transient Transfection Assay—COS cells were grown to 40% confluence and transfected by calcium phosphate method as described previously (2). After transfection, cells were shocked with glycerol and generally maintained for 2 days before harvesting. CAT assays were performed as described elsewhere (2).

In Vivo Protein Binding Assay—The in vivo binding assay was performed essentially as described elsewhere (12). COS cells were cotransfected with expression vectors pMT2/MEF2A and pRSV/MASH1. Whole cell protein extracts were prepared as described above. The protein extracts were incubated with anti-MASH1 antibody in NET buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) in the presence of protein A beads. The immunoprecipitated complexes were then separated on a SDS-PAGE and probed with anti-MEF2A antibody on Western blot analysis as described above. For sequential immunoprecipitation, the experiment was carried as described elsewhere (12). The associated proteins from the initial immunoprecipitation, released by boiling SDS, was reprecipitated with secondary antibodies, and the final precipitates were analyzed by SDS-PAGE.

In Vitro Protein Binding Assay—The in vitro binding assays were performed as previously described (1, 12). <sup>35</sup>S-MASH1 or MEF2A generated by in vitro translation was incubated with bacterially expressed GST MEF2A or GSTMASH1 by incubation to glutathione-agarose beads, respectively, in NET buffer at 4 °C with rocking for 3 h. The protein complexes bound to the agarose beads were washed five times with the same buffer. The resulting precipitated complexes and supernatants were analyzed by SDS-PAGE, and the proteins were visualized by autoradiography.
Fig. 1. Expression of MEF2A and MASH1 in P19 cells. A, Western blot analysis of the induction of MEF2A upon P19 neuronal differentiation. Lane 1 is a MEF2A control. Protein extracts from P19 cells before (lane 2) and after retinoic acid treatment (lanes 3–7, 1, 2, 3, 4, and 5 days post retinoic acid, respectively) were analyzed with an anti-MEF2A specific antibody. Protein size standards are indicated in kilodaltons and the arrow marks the position of MEF2A. B, indirect immunofluorescence showing the coexpression of MASH1 and MEF2A in differentiating P19 cells. P19 cells were treated with retinoic acid and allowed to aggregate for 1 day. After culture for another 2 days in the absence of retinoic acid, cells were trypsinized and cultured for 1 day before being assayed for the presence of MEF2A and MASH1 with a polyclonal antibody to
the two markers (Fig. 1 throughout the differentiation process or expressed just one of in culture which were not stained with either antibody after retinoic acid addition to the cells (13). To determine if MEF2A-like phenotype (9–11). Early in differentiation, MASH1 expression of various neural markers and acquisition of a neuronal phenotype. Immunostaining of retinoic acid-treated cells with antibodies to MASH1 and MAP2 was performed at differentiation. Double immunostaining of P19 cells with antibodies to MASH1 and MEF2A revealed cells positive for marker MAP2 and MASH1 (Fig. 1a). Although there were also cells in culture which were not stained with either antibody throughout the differentiation process or expressed just one of the two markers (Fig. 1b), panel b), a higher percentage of MASH1-positive cells was found at the early stage of differentiation. At this stage, a high percentage of the MASH1-positive cells were also positive for MEF2A (70%) (Fig. 1b, panel a and Table I). The percentage of MASH1-positive cells declined as cells became more differentiated while the number of MEF2A-positive cells increased. At later stages of the process, only about 5% of MEF2A-positive cells were also MASH1-positive (Table I).

Cooperative Transactivation of Gene Expression by MASH1 and MEF2A in Transient Transfection Assay—The observation that MASH1 and MEF2A are coexpressed in differentiating P19 cells raised the possibility that neuronal specific MASH1 might cooperate with MEF2A to activate gene expression in a manner similar to the cooperative roles of MEF2 and MyoD gene families in myogenesis (1). To test this hypothesis, we examined MEF2A and MASH1 alone or in combination in a transient transfection assay for their ability to stimulate expression of a CAT reporter gene containing both a MEF2 and a MyoD promoter (TK or embryonic myosin heavy chain genes) were examined MEF2A and MASH1 alone or in combination in a transient transfection assay for their ability to stimulate expression of a CAT reporter gene containing both a MEF2 and a MyoD promoter (TK or embryonic myosin heavy chain genes). The relative degree of activation over background corrected for efficiency of transfection is indicated. The experiments were repeated four times, and data from a typical experiment are shown.

### Table I

| Days after initial RA treatment | MEF2A/MASH1 | MASH1/MEF2A |
|-------------------------------|-------------|-------------|
| 3                             | 70%         | 60%         |
| 4                             | 18%         | 5%          |

### RESULTS

Coordinated Expression of MASH1 and MEF2A during P19 Differentiation—In initial studies we found that MASH1 and MEF2A were coordinately expressed during neural differentiation. When teratocarcinoma P19 cells are exposed to retinoic acid, they differentiate along a neural lineage, as indicated by expression of various neural markers and acquisition of a neuron-like phenotype (9–11). Early in differentiation, MASH1 mRNA was transiently expressed and first detected 2 days after retinoic acid addition to the cells (13). To determine if MEF2A was also induced during P19 neural differentiation, we examined protein extracts for the presence of MEF2A by Western blot analysis using an isoform-specific antibody to MEF2A. Analysis of MEF2A expression by Western blot assay revealed that MEF2A protein was not present before differentiation but was induced concomitantly with MASH1, appearing around day 2 after retinoic acid stimulation (Fig. 1A). However, unlike the transient expression of MASH1, MEF2A showed a prolonged pattern of expression starting on day 2 and continuing at high levels 5 days after the exposure to the inducer. Since the induction of MEF2A coincides with that of MASH1 in P19 cell cultures, we next asked whether MEF2A and MASH1 were coexpressed in the same cells during the initial stages of neural differentiation. Double immunostaining of P19 cells with antibodies to MASH1 and MAP2 was performed at differentiation day 2. Indirect immunofluorescence clearly identified differentiating cells which express both MASH1 and MAP2 (Fig. 1b, panel c), confirming the neuronal identity of the MASH1-positive cells. Immunostaining of retinoic acid-treated cells with antibodies to MASH1 and MEF2A revealed cells positive for both proteins (Fig. 1b, panel a). Although there were also cells in culture which were not stained with either antibody throughout the differentiation process or expressed just one of the two markers (Fig. 1b, panel a), a higher percentage of MASH1-positive cells was found at the early stage of differentiation. At this stage, a high percentage of the MASH1-positive cells were also positive for MEF2A (70%) (Fig. 1b, panel a and Table I). The percentage of MASH1-positive cells declined as cells became more differentiated while the number of MEF2A-positive cells increased. At later stages of the process, only about 5% of MEF2A-positive cells were also MASH1-positive (Table I).

Cooperative Transactivation of CAT reporter gene by MEF2A and MASH1. A diagram of the prototypical reporter genes for these cotransfection experiments showing a MEF2 binding site and an E box in front of the basal TK gene promoter linked to the bacterial CAT coding sequence: (the empty box) no binding site; E, the E box; M, the MEF2 binding site; (j) a mutated site. B, CAT activity from CV1 cells transfected with MT2MEF2A and/or pRSV/MASH1 together with different reporter gene constructs. The relative degree of activation over background corrected for efficiency of transfection is indicated. The experiments were repeated four times, and data from a typical experiment are shown.

**FIG. 2.** Cooperative transactivation of CAT reporter gene by MEF2A and MASH1. A, diagram of the prototypical reporter genes for these cotransfection experiments showing a MEF2 binding site and an E box in front of the basal TK gene promoter linked to the bacterial CAT coding sequence: (the empty box) no binding site; E, the E box; M, the MEF2 binding site; (j) a mutated site. B, CAT activity from CV1 cells transfected with MT2MEF2A and/or pRSV/MASH1 together with different reporter gene constructs. The relative degree of activation over background corrected for efficiency of transfection is indicated. The experiments were repeated four times, and data from a typical experiment are shown.

**TABLE I**

| Binding Site | Basal Promoter | CAT |
|-------------|----------------|-----|
| E           | M              | E   |
| E           | M              | E-M |
| E           | M              | E-M |
| E           | M              | E-M |

Z. Mao and B. Nadal-Ginard, unpublished data.
between MEF2 and bHLH protein of the MyoD family (1, 2). Cotransfection of CV1 cells with the TKCAT reporter construct and the expression constructs for either MEF2A or MASH1 produced no significant change in reporter gene activity (Fig. 2B). However, coexpression of both MEF2A and MASH1 together resulted in a significant increase in CAT reporter activity. That this induction was due to interaction of the respective transcription factors with their DNA binding sites was supported by the demonstration that point mutations in either the E box or the MEF2 binding site, which in DNA mobility shift assays disrupted MASH1 or MEF2A binding, respectively (data not shown), rendered the reporter gene unresponsive to both MASH1 and MEF2A individually or in combination (Fig. 2B).

In Vivo Physical Interaction between MASH1 and MEF2A—The observed functional cooperativity between MASH1 and MEF2A raised the possibility that these factors might physically interact with each other in vivo. To test this possibility, we transfected COS cells with expression vectors encoding MASH1 and MEF2A and performed immunoprecipitation experiments (12) using anti-MASH1 antibodies followed by Western blot analysis using a MEF2A-specific antisemur. Antibodies against MASH1 specifically immunoprecipitated MEF2A as indicated by Western blots with anti-MEF2A antibodies (Fig. 3A). MEF2A was not detected when control antibody was used for the immunoprecipitation step. Furthermore, of the two MEF2A species expressed in cells, only the faster migrating MEF2A band was coprecipitated with MASH1. The slower migrating MEF2A species, although present in the cellular protein extract prior to immunoprecipitation, was not detected in the MASH1 immunoprecipitated complex. In a reverse experiment, immunoprecipitation of 35S-Met-labeled cell extracts was carried out with first either anti-MEF2A or preimmune serum and followed by sequential immunoprecipitation with anti-MASH1 antibodies. Only anti-MEF2A antibody specifically immunoprecipitated MASH1 (Fig. 3B). These findings provide evidence that MASH1 and MEF2A physically interact within cells and raise the possibility that a post-transcriptional modification of MEF2A regulates its interaction with MASH1.

Determination of the Domains of MASH1 and MEF2A Involved in the Protein-Protein Interaction by in Vitro Binding—To identify the domains of the MASH1 and MEF2A proteins that are involved in the protein-protein interaction, we first established an in vitro binding assay using bacterially expressed glutathione S-transferase (GST) fusion protein (Fig. 4A) (12). 35S-MASH1 generated by in vitro translation was incubated with GST-MEF2A fusion protein bound to agarose beads. The protein bound to the beads was resolved by SDS-PAGE; lane 1, MASH1 translated in vitro as control; lane 2, protein remaining in the supernatant after incubation with GST; lane 3, protein bound by GST; lane 4, protein bound by GST-MEF2A. Protein standards are indicated in kilodaltons and the arrow marks the position of MASH1. B, mapping the domain of MASH1 involved in binding to MEF2A factors. GSTMASH1bHLH was incubated with 35S-MEF2A generated by in vitro translation and the bound proteins were analyzed as in A. Lane 1, MEF2A control; lane 2, that protein remained in the supernatant after incubation with GST; lane 3, protein bound by GST; lane 4, protein bound by GST-MASH1bHLH. The arrow marks the position of MASH1.

C, mapping the domain of MEF2A involved in binding to MASH1. 35S-MEF2A deletion mutants (DW) were incubated with GST-MASH1bHLH. Lanes 1–3 are in vitro binding between GSTMASH1bHLH and DW 57–322 (lane 1, DW 57–322 control; lane 2, protein bound by GST; lane 3, protein bound by GST-MASH1bHLH). Lanes 4–15 are in vitro binding between GSTMASH1bHLH and various MEF2A deletion mutants. Lanes 4–6 are in vitro translated deletion mutants (lane 4, DW 1–56; lane 5, DW 1–87; lane 6, DW 1–131). Lanes 7–15 are binding assays of MEF2A deletion mutants (lanes 7, 10, and 13, DW 1–56; lanes 8, 11, and 14, DW 1–81; lanes 9, 12, and 15, DW 1–131), with either GST-MASH1bHLH (lanes 7–9, pellets from the incubation; lanes 10–12, MEF2A control from the incubation; and lanes 13–15, diagrams of the domains required for MASH1 and MEF2A to interact with each other as determined by the in vitro binding assays.

D, diagrams of the domains required for MASH1 and MEF2A to interact with each other as determined by the in vitro binding assays.
DISCUSSION

We show here that MASH1 and MEF2A are coordinately induced and coexpressed in P19 cells during their differentiation along a neuronal lineage. In transient transfection assays, MASH1 and MEF2A cooperatively activate gene expression. The basis of the cooperativity appears to be a specific physical interaction between MASH1 and MEF2A mediated through their conserved bHLH motif and MADS domain, respectively. These findings suggest that these two classes of transcription factors may function as a complex to regulate transcription of genes important for neurogenesis.

These results raise questions regarding the natural gene targets of MASH and MEF2A factors during neurogenesis. Putative MASH1 and MEF2 binding sites are present in the promoters of neural specific genes such as neurological SCL1 and -2, brain-specific type II sodium channel, and neural cell adhesion molecule NCAM (17-21). Among them, it is particularly interesting to note that a MEF2 site and an E box are present in the regulatory region of NSC11 and -2 genes, making them candidate targets of the cooperative effects of MASH1 and MEF2A.

The observation that MEF2A and MASH1 interact both functionally and physically may have broad implications for understanding aspects of neural development and differentiation. Several neural specific bHLH proteins and different isoforms of MEF2 are present in various regions of the central and peripheral nervous systems during very early stages of mouse development and also in the adult mouse brain (5, 6, 7, 22, 23). Taken together with the findings described in this report, these observations raise the possibility that neural specific bHLH proteins may interact with MEF2 factors at various stages of neural development. The MASH1 and MEF2A interaction may be an indication of a more general interaction between various members of these two families of transcription factors. Consistent with this idea is the finding that the highly conserved MADS and bHLH domains are required for the interaction between MASH1 and MEF2A and the observation that other neuronal specific bHLH proteins can also specifically interact with different isoforms of MEF2A. Therefore, it is possible that different combinations of neural specific bHLH proteins and MADS domain-containing proteins may cooperate to regulate gene expression at different stages of development. Such a combinatorial mechanism would allow the production of a large number of specific and distinct arrays of proteins beginning with a relatively small number of transcription factors.

Given that an interaction between MEF2A and the bHLH protein MyoD has recently been shown to be required for muscle development, our observations predict that certain aspects of the basic mechanisms of transcriptional control of lineage-specific determination may be conserved during development of the mammalian muscle and neuronal system. In addition, normal myogenesis and neurogenesis are dependent upon the presence of functional retinoblastoma protein Rb (24-26).

Taken together, all of these observations suggest a general model for myogenic and neurogenic gene expression as well as for cell lineage determination which requires the interaction of at least three families of regulatory factors with increasingly restricted patterns of gene expression. In this scheme Rb is the universal permissive factor, MEF2 with its multiple gene products is restricted to a limited and well defined set of cell types, while the myogenic and neurogenic bHLH proteins provide the required cell-type specificity.

Acknowledgments—We thank Dr. Michael E. Greenberg for his timely advice, generous help, and interest in various aspects of the work. This work would not have been possible without his tremendous support, for which we are indebted to Drs. D. J. Anderson and J. E. Johnson for providing the MASH1 expression construct and antibody, to S. Kaushal for various MEF2 constructs, and to V. Mahdavi for helpful discussions.

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Functional and Physical Interactions between Mammalian Achaete-Scute Homolog 1 and Myocyte Enhancer Factor 2A
Zixu Mao and Bernardo Nadal-Ginard

J. Biol. Chem. 1996, 271:14371-14375.
doi: 10.1074/jbc.271.24.14371

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