cMeso-1, a Novel bHLH Transcription Factor, Is Involved in Somite Formation in Chicken Embryos

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The segmentation of somites from the paraxial mesoderm is a crucial event in vertebrate embryonic development; however, the mechanisms underlying this process are not well understood. In a yeast two-hybrid screen we have identified the novel basic-helix-loop-helix (bHLH) protein cMeso-1 which is expressed in the presomitic mesoderm of early chicken embryos. Initially the gene is activated in the epiblast and transcripts concentrate later in and around the primitive streak. When the segmental plate is laid down the cMeso-1 expression domain successively retracts toward the caudal end but a second domain appears in bilateral stripes in the anterior paraxial mesoderm. This highly dynamic domain of cMeso-1 transcripts demarcates the area immediately posterior to the next prospective pair of somites in cyclic waves which apparently correspond to the formation of new somites. Loss of cMeso-1 function by antisense RNA or oligonucleotides results in severe attenuation of somitogenesis suggesting that it plays an important role in setting up the segmentation process. The dynamic and periodically reiterated expression of cMeso-1 along the anteroposterior axis is not dependent on anterior structures or the propagation of a signal along the anteroposterior axis but seems to follow an intrinsic patterning program which is already set up in the segmental plate.© 1998 Academic Press

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INTRODUCTION

One of the crucial events during vertebrate development is the segmentation of paraxial mesoderm which gives rise to somites, the first observable metameric units in the embryo. Paraxial mesoderm develops on both sides of the notochord and neural tube in a rostrocaudal direction when Hensen’s node and the primitive streak retract caudally (Slack, 1991). Somitogenesis starts in the cranial region of the embryo and is accompanied by considerable morphogenetic changes of the tissue architecture within the mesoderm. The initially mesenchymal cells of the paraxial mesoderm undergo compaction and transition from mesenchyme to a spherical epithelium which surrounds the somitocoel that is filled with mesenchymal cells. These epithelial somites are subsequently subdivided into the ventral mesenchymal sclerotome and the dorsal epithelial dermomyotome. The sclerotome will develop into the axial skeleton, while the dermomyotome further subdivides into the myotome and dermatome giving rise to epaxial musculature and connective tissue of the dermis, respectively. The broad contribution of somitic cells to multiple tissues implies that mutations in genes which are expressed in somites and are functionally involved in the formation of the skeleton and musculature will have severe effects on body morphology (Tam and Trainor, 1994). Genes and molecular mechanisms underlying cell type specification of somitic derivatives, such as the sclerotome and dermomyotome (Bober et al., 1994; Fan and Tessier-Lavigne, 1994; Fan et al., 1995; Olson and Klein, 1994; Burgess et al., 1995; Cserjesi et al., 1995; Pourquie et al., 1996; Winnier et al., 1997), have been studied in great detail and their actions are particularly well understood for the development of skeletal muscles (reviewed by Arnold and Braun, 1996). In contrast, molecular information on the process of somitogenesis itself and the maintenance of segment borders is rather limited (Yamaguchi, 1997).

Although the presomitic mesoderm appears unsegmented under the light microscope, several lines of evidence suggest that the paraxial mesoderm is prepatterned in so-called somitomeres (Packard and Jacobson, 1976; Pack-
ard, 1978; Meier, 1979; Jacobson and Meier, 1986). Thus, the segmentation program may be established in mesoderm prior to overt morphological appearance of segment borders. It has been demonstrated recently that the vertebrate members of the Notch/Delta gene families may play a role in the segmentation process. Delta and Notch proteins interact with each other and are thought to be required for cell fate decisions (Corbin et al., 1991) as well as epithelial-mesenchymal transitions (Hartenstein et al., 1992; Tepass and Hartenstein, 1995). The mouse genes Notch-1 and Notch-2 are expressed in the paraxial mesoderm (Del Amo et al., 1992; Lardelli and Lendahl, 1993; Reaume et al., 1992) and mice mutants lacking Notch-1 exert disorganized and delayed somitogenesis (Conlon et al., 1995). The mouse Delta-like gene DII1 is expressed in the presomitic mesoderm and in the posterior half of somites (Bettencourt et al., 1995). DII1 mutant embryos lose the cranio-caudal polarity of somites and the ability for epithelialization, although the primary metameristic pattern of the mesoderm seems to be maintained (Hrabe de Angelis et al., 1997). Paraxis, a basic helix-loop-helix protein, is also expressed in paraxial mesoderm and somites (Burgess et al., 1995). Mice carrying a homozygous null mutation of the paraxis gene are unable to form epithelial somites and show disruption of correct patterning of tissues derived from the paraxial mesoderm (Burgess et al., 1996).

In an attempt to identify additional genes which may play a role in mesoderm segmentation we isolated a novel chicken basic helix-loop-helix (bHLH) protein, referred to as cMeso-1. This gene is first expressed in the epiblast of Hamburger-Hamilton (HH) stage 5 chicken embryos and subsequently concentrates in and around the primitive streak. During further development cMeso-1 expression retreats caudally and an additional dynamic expression domain arises as a bilateral stripe in the anterior presomitic mesoderm. This cMeso-1 domain seems to oscillate with the formation of new somites and always precedes the actual segmentation process. Application of antisense RNA or antisense oligonucleotides to chicken embryos severely

![FIG. 1A.](image)

**FIG. 1A.** Nucleotide and deduced amino acid sequence of cMeso-1 cDNA. The bHLH region is marked by a gray box and the polyadenylation signal is underlined.

![FIG. 1B.](image)

**FIG. 1B.** Comparison of the cMeso-1 bHLH domain with that of other bHLH proteins. Amino acid identities are indicated by dashes. The compared sequences are MesP2 by Saga et al. (1997); MesP1 by Saga et al. (1996); paraxis by Barnes et al. (1997); M-twist by Blanar et al. (1995); and dHand by Srivastava et al. (1995).
The posterior domain regresses caudally in parallel to the retracting primitive streak (B–D). Accumulation of cMeso-1 transcripts in bilateral stripes at the level of Hensen's node (A) which later increases in intensity in the anterior presomitic mesoderm (5 (A), 6 (B), 7 (C), and 8 (D) embryos are shown. First transcripts are detected in and around the primitive streak. Initially faint staining is attenuated somitogenesis suggesting that cMeso-1 plays an important role in the segmentation process.

MATERIALS AND METHODS

Yeast two-hybrid screening and isolation of cMeso-1 cDNA. cDNA of HH stage 8 chicken embryos was cloned into the HybriZAP vector (Stratagene, Heidelberg, Germany) to generate a primary lambda library. The library was amplified and converted by in vivo excision to a pAD-GAL4 plasmid library for expression in yeast. The bait vector Gal4-BD-E12 was obtained by ligation of the PCR-generated EcoRI/BamHI cDNA fragment. Transcripts of approximately 1.2 kb were detected exclusively in the presomitic mesoderm of stage 12 embryos (top). For the RNA loading control the blot was hybridized with a chicken GAPDH probe (bottom).

Northern blot analysis. Total RNA from chicken embryonic tissues was isolated by the guanidinium thiocyanate method (Auffray and Rougeon, 1980). The RNA was glyoxylated, separated on a 1% agarose gel, and transferred to a Nylon membrane (PALL Biodyne A). Hybridization was carried out in 50% formamide, 5× Denhardt’s, 0.1% SDS, 50mM sodium phosphate, 5× SSPE, and 50 μg/ml denatured salmon sperm DNA at 42°C. A 798-base-pair EcoRI/BamHI fragment was 32P labeled by random priming to a specific activity of 1 × 106 dpm/μg. A GAPDH probe served as RNA loading control.

Whole-mount in situ hybridizations. Antisense and sense RNA probes for cMeso-1 and cFKH-1 were labeled with digoxigenin-11-UTP using T7 polymerase and the pAD-GAL4-cMeso-1 plasmid linearized with EcoRI. Double in situ hybridizations were performed with dll and paraxis probes labeled with fluorescein and detected with anti-fluorescein antibody using Fast Red as substrate. Chick embryos of various stages were fixed with 4% paraformaldehyde/PBS at 4°C overnight. Whole-mount in situ hybridizations of chicken embryos between HH stages 5 and 8 with a cMeso-1 probe. Dorsal views of stages 5 (A), 6 (B), 7 (C), and 8 (D) embryos are shown. First transcripts are detected in and around the primitive streak. Initially faint staining is also observed in bilateral stripes at the level of Hensen's node (A) which later increases in intensity in the anterior presomitic mesoderm (B–D). The posterior domain regresses caudally in parallel to the retracting primitive streak (B–D). Accumulation of cMeso-1 transcripts in the anterior domain precedes somite formation and becomes undetectable in epithelial somites. Transverse vibratome sections (30 μm) through the posterior expression domain of stage 5 (E and F) and 6 (H) embryos indicate that cMeso-1 transcripts first accumulate in the epiblast (E) and subsequently in the mesoderm (F–H). A section through the anterior domain shows the cMeso-1 transcript in the mesoderm (G). The level of sections in indicated. hn, Hensen’s node; ps, primitive streak; hf, head fold; pso, prospective somite; m, mesoderm; en, endoderm; e, ectoderm; ep, epiblast; pf, primitive fold.

FIG. 3. Whole-mount in situ hybridizations of chicken embryos between HH stages 5 and 8 with a cMeso-1 probe. Dorsal views of stages 5 (A), 6 (B), 7 (C), and 8 (D) embryos are shown. First transcripts are detected in and around the primitive streak. Initially faint staining is also observed in bilateral stripes at the level of Hensen's node (A) which later increases in intensity in the anterior presomitic mesoderm (B–D). The posterior domain regresses caudally in parallel to the retracting primitive streak (B–D). Accumulation of cMeso-1 transcripts in the anterior domain precedes somite formation and becomes undetectable in epithelial somites. Transverse vibratome sections (30 μm) through the posterior expression domain of stage 5 (E and F) and 6 (H) embryos indicate that cMeso-1 transcripts first accumulate in the epiblast (E) and subsequently in the mesoderm (F–H). A section through the anterior domain shows the cMeso-1 transcript in the mesoderm (G). The level of sections in indicated. hn, Hensen’s node; ps, primitive streak; hf, head fold; pso, prospective somite; m, mesoderm; en, endoderm; e, ectoderm; ep, epiblast; pf, primitive fold.

FIG. 4. cMeso-1 expression in stage 9 to 15 embryos shown by whole-mount in situ hybridization. Dorsal views of embryos indicate that cMeso-1 transcripts retract to the caudal end of the primitive streak, while bilateral stripes of cMeso-1 positive cells appear in the anterior paraxial mesoderm. These stripes of cMeso-1 expression move in an anteroposterior direction in front of the newly forming somites and almost reach the tailbud in a stage 15 embryo (A–D). Higher power magnification of C is shown in D to demonstrate the position of cMeso-1 positive cells in yet an unsegmented mesoderm posterior to the region which will form the next somites. A shows an embryo in which one stripe has shifted toward the anterior end of the presomitic mesoderm, while more caudally the next stripe has already activated cMeso-1 expression. Vibratome sections at the levels indicated in B and C confirm that transcripts are limited to the presomitic mesoderm with higher expression in cells of the periphery which are destined to become the epithelium (E and F). nc, notochord; nt, neural tube; pm, presomitic mesoderm; pso, prospective somite; ps, primitive streak.
tions and vibratome sections were performed as described previously (Buchberger et al., 1996).

**Embryo culture and treatment with antisense oligonucleotides or RNA.** Fertilized eggs from white Leghorn chickens were incubated at 38.5°C for about 45 h when embryos were removed from the eggs, carefully cleaned from yolk in Tyrode’s solution, and staged by counting somites as described by Hamburger and Hamilton (1951). Embryos with 7 to 14 somites were used for treatment with antisense nucleic acids.

For cultivation in vitro embryos were placed on agar dishes (0.6% Bacto agar, 0.7% NaCl, 50% egg albumin) with the dorsal side up and incubated at 38.5°C. Antisense oligonucleotides were synthesized as phosphorothioate derivatives, purified by HPLC, and dissolved in sterile PBS to a final concentration of 80 μM. Two microliters of sense or nonsense oligonucleotides was added directly to the embryo at the start of the experiment and every 2 h thereafter. The treatment was terminated after 6 h by fixation of embryos with 4% paraformaldehyde in PBS overnight prior to whole-mount in situ hybridization. A control group of animals was treated with PBS alone. Numbers of somites were counted at the start and the end of the experiments. Normal development of head and heart was monitored to exclude general toxicity of the oligonucleotides and adverse effects of in vitro cultivation. Treatment with antisense RNA in ovo was performed on embryos which were contrasted by injection of India-Ink solution (1:5 dilution in PBS).

In vitro transcribed antisense or sense RNA (5 μg) was incubated for 15 min with 300 μl Superfect transfection reagent (Qiagen, Hilden, Germany) and 3–5 μl was injected through the vitelline membrane directly onto the embryos. A control group of embryos received Superfect transfection reagent without RNA. The eggs were sealed with polythene foil and incubated for a further 6 h. Pairs of somites and normal development were scored as described above.

The following oligonucleotides were used: cMeso-1 antisense oligo, 5′-GTGGCAGGAACCGGCTCAAGCTCC-3′; cMeso-1 sense oligo, 5′-GGAGCTGCACGGGGTTCCTGCCAC-3′; and cMeso-1 nonsense oligo, 5′-GTCCGACCGGTAGCCTGTA-CG-3′. All selected oligonucleotides did not show significant homology with other gene sequences contained in GenBank databases. For statistical evaluation all data were subjected to Student’s t test and expressed as mean ± SEM.

**Surgical manipulation of embryos.** Stage 9 to 12 chicken embryos were removed from the eggs, cleaned from yolk in PBS, and transversely dissected with tungsten needles at various axial levels as illustrated in Fig. 7. Anterior and posterior parts of the embryos were cultivated separately on agar dishes (0.6% Bacto agar, 0.7% NaCl, 50% egg albumin) for 8 to 20 h during which somitogenesis was monitored. The explants were fixed in 4% paraformaldehyde/PBS for in situ hybridization with cMeso-1, cFKH-1, or paraxis probes.

**RESULTS**

**Isolation of the chicken cDNA cMeso-1 encoding a novel bHLH transcription factor.** To identify novel bHLH proteins expressed during early chick development we employed the yeast two-hybrid system using the conserved bHLH dimerization domain of the ubiquitously expressed transcription factor E12 as bait. A cDNA library of HH stage 8 chicken embryos was screened for E12 dimerization partners. From a total of 1 × 10⁶ independent transformants we obtained 23 clones which grew under Leu/Trp/His selection and also expressed β-galactosidase. These clones were sequenced and the results subjected to a Blast search of the GenBank database. Among several known bHLH proteins, such as paraxis, eHand, and dHand, one partial cDNA clone of 0.9 kb encoded a novel bHLH domain. To isolate the full-length cDNA the HH stage 8 embryonic chick library was rescreened. The longest clone contained an insert of 1082 nucleotides with an open reading frame of 828 nucleotides encoding 287 amino acids (Fig. 1A). This clone was designated cMeso-1 for its mesodermal expression (see below). A typical polyadenylation signal (AATAAA) was located 13 nucleotides upstream of the poly(A) stretch. The first methionine codon was preceded by the Kozak consensus sequence (Kozak, 1997) suggesting that it represented the translation initiation site. Comparison of the cMeso-1 bHLH domain with those of known proteins revealed 76.4% and 74.5% identity with the recently isolated mouse genes MesP2 and MesP1 (Saga et al., 1996, 1997) and homology to paraxis (Barnes et al., 1997), M-twist (Blanar et al., 1995), and dHand (Srivastava et al., 1995) of 49, 47, and 45%, respectively (Fig. 1B). Essentially no homology to these or any other proteins was found outside of the bHLH domain at the nucleotide or protein level. Based on this limited sequence similarity it seems questionable whether cMeso-1 represents the chicken orthologue of mouse MesP1 or MesP2, as bHLH domains of true orthologues of chicken and mouse bHLH proteins exhibit homologies between 94 and 100% throughout the entire coding region as recently exemplified for paraxis (Sosic et al., 1997). Thus, cMeso-1 may be a new member of the MesP-related family of transcription factors.

**cMeso-1 transcripts accumulate in the presomitic mesoderm and seem to demarcate the area of the next to form somites during early chick development.** First evidence that embryonic cMeso-1 expression may be restricted to paraxial mesoderm came from Northern blot analysis of embryonic chicken tissues which revealed cMeso-1 transcripts of about 1.2 kb in the presomitic mesoderm but in no other tissue (Fig. 2). To determine the spatiotemporal expression pattern in more detail whole-mount in situ hybridizations were performed on chicken embryos with a digoxigenin-labeled cMeso-1 antisense RNA probe. First transcripts were detected in cells surrounding the primitive streak of stage 5 embryos (Fig. 3). This expression domain was limited to the medial and posterior portion of the embryo and appeared much wider at its anterior margin. During subsequent development the cMeso-1 expression domain became more concentrated to the medial mesoderm and gradually retracted toward the caudal end in parallel to the regression of the primitive streak (Figs. 3A–3D). A second, initially weak cMeso-1 expression domain became visible in two bilateral stripes at the anterior end of the arising paraxial mesoderm approximately at the level of Hensen’s node (Fig. 3). With ongoing somitogenesis this domain increased in intensity and moved in a rostro-caudal direction remaining at an approximately equal dis-
tance posterior to the newly formed somites (Figs. 3B–3D). Transversal vibratome sections through the anterior and posterior domain in early stage embryos demonstrated that CMeso-1 transcripts were initially present in the epiblast and were later limited to the arising mesoderm (Figs. 3E–3H). The highly dynamic mode of CMeso-1 expression in the paraxial mesoderm became even more apparent when older embryos were analyzed. At stages 9 to 15 the posterior expression domain approached the tailbud, while the ante-
rior stripes continued to reappear in consistent waves throughout the somite formation period (Figs. 4A–4D). No CMeso-1 expression was found so far in later tissues. Inter-
estingly, in most embryos analyzed the anterior CMeso-1 domain appeared as one pair of bilateral stripes caudal to the last formed somites leaving a gap of approximately one somite diameter (Figs. 4B–4D). In a fraction of embryos two CMeso-1 positive stripes were observed; one, immediately next to the last formed somites and one at a distance of approximately one prospective somite (Fig. 4A). Embryos without any CMeso-1 positive stripes were never observed. To better define the precise location of CMeso-1 transcripts within the presomitic mesoderm double in situ hybridiza-
tions were performed with the CMeso-1 probe together with either a dll (Delta-like)-specific or paraxis-specific probe (Fig. 5). Both of these genes are expressed in distinct patterns in the paraxial mesoderm and in somites (Bettenhausens et al., 1995; Burgess et al., 1995). Whole-mount hybridizations and transversal vibratome sections confirmed that CMeso-1 transcripts accumulate in register with prospective somites, initially posterior to the next somite forming region whose anterior border is defined by dll expression (Fig. 5A). Thus, dll expression overlaps with CMeso-1 but apparently precedes it. Similarly, CMeso-1 expression also overlaps with paraxis expression but the paraxis domain extends more caudally, suggesting that it also precedes CMeso-1 activation (Figs. 5C and 5D). Signifi-
cantly, however, the anterior and posterior borders of CMeso-1 positive stripes in relation to dll and paraxis expression, respectively, were somewhat variable indicating the dynamic nature of the CMeso-1 domain during the somite formation cycle (Figs. 5A–5D). Moreover, in some embryos two CMeso-1 stripes were observed with the ante-
rior stripe appearing less then one somite in length (Figs. 5E and 5F). This observation suggests that CMeso-1 transcripts disappear from the posterior of the next-to-form somite prior to the anterior portion, while the next caudal expression domain has already begun to accumulate CMeso-1 transcripts. It should be noted that we never observed CMeso-1 expression within already segmented somites. Transverse sections through the overlapping expression domains revealed that CMeso-1 mRNA primarily accumu-
lates at the dorsal and ventral sites of the presomitic mesoderm unlike the transcripts for dll and paraxis which appear more evenly distributed throughout the medial mesoderm. In summary, CMeso-1 transcription appears to be initiated in the paraxial mesoderm that is in preparation of somite formation but the gene is active only until somites actually begin to form.

CMeso-1 antisense RNA or antisense oligonucleotides attenuate somitogenesis in chicken embryos. As an at-
temt to explore the possible importance of CMeso-1 for somitogenesis we took a loss of function approach by applying antisense RNA or antisense oligonucleotides to chicken embryos. In a series of experiments antisense RNA dissolved in SuperFect reagent was injected through the vitelline membrane directly on stage 10 embryos in ovo and incubation was continued for an additional 6 h. The number of somites was counted at the time of RNA injection and 6 h later. Under this treatment we observed no abnor-
mal development of head and heart structures suggesting that the reagents were not generally toxic. Control animals injected with SuperFect reagent alone or with sense RNA developed on average 3.4 (±0.22) somites within 6 h (Fig. 6) similar to embryos that were not treated at all (data not shown). In contrast, chicken embryos injected with CMeso-1 antisense RNA formed only one additional pair of somites during the same period. This significant difference strongly suggested that CMeso-1 is necessary for the forma-
tion of somites. To avoid unspecific effects which may possibly be exerted by antisense RNA a second series of experiments was performed using antisense oligonucleo-
tides on chicken embryos which were cultivated on agar dishes. Oligonucleotides dissolved in PBS were applied to the embryos every 2 h for a total of 6 h. The number of newly formed somites was scored as described above. We also checked for normal development of head and heart. As shown in Fig. 6B, approximately one pair of somites de-
veloped every 2 h in the control groups that had received either PBS alone or PBS plus randomized nonsense oligonucleo-
tides. The numbers of 3.5 ± 0.34 and 3.00 ± 0.25 newly formed somites in embryos treated with PBS or nonsense oligonucleotides were in good agreement with those ob-
erved in the in vivo experiments. Application of CMeso-1 antisense oligonucleotides to the embryos resulted in sig-
nificant attenuation of somitogenesis with only one addi-
tional pair of somites formed in 4 h and almost complete arrest of somitogenesis thereafter (Fig. 6B). Identical results were obtained with oligonucleotide injections in ovo (data not shown). In addition to the suppression of somitogenesis, both antisense RNA and antisense oligonucleotides also led to posterior truncations of the body axis in the majority of embryos (Fig. 6H, compare embryos treated with antisense and nonsense oligonucleotides). To control the effect of antisense oligonucleotides on the level of CMeso-1 tran-
scripts whole-mount in situ hybridizations were performed on embryos fixed 1, 3, or 6 h following treatment. Severe reduction of CMeso-1 staining was already observed 1 (data not shown) and 3 h after the addition of antisense oligonucleotides (Fig. 6D) with essentially no transcripts remaining after 6 h (Fig. 6E). Randomized nonsense oligonucleotides had no effect on the level of CMeso-1 transcripts within 6 h (Fig. 6C). The specificity of oligonucleotide treatment was further controlled by hybridization with the cFKH1 probe.
FIG. 5. Double in situ hybridizations of stage 11 to 15 chick embryos with cMeso-1 probe (blue) and either dll (delta-like) probe (red in A, B, and E) or paraxis probe (red in C, D, and F). Dll staining in the presomitic mesoderm defines the anterior border of the segmental plate and illustrates the dynamic position of cMeso-1 expression relative to this border in different embryos (A, B, and E). Arrows indicate the positions of the last formed somites; arrowheads mark the anterior end of the unsegmented paraxial mesoderm. The relationship of paraxis and cMeso-1 expression domains is shown in C, D, and F. Transverse sections were taken through the region of overlapping expression on a vibratome.
which a transcript of the forkhead 1 gene in the presomitic mesoderm and somites (Buchberger et al., 1998). As shown in Fig. 6F, cFKH1 transcript accumulation was unaffected by the 6-h treatment with antisense oligonucleotides. The same treatment also did not affect the normal accumulation of paraxis transcripts in the presomitic mesoderm and in somites (Fig. 6G). Taken together these results provide evidence that antisense oligonucleotides specifically inhibit the expression of cMeso-1 in the embryo. The lack of cMeso-1 then leads to impairment of somite formation. Whether under these conditions somitogenesis was completely blocked or only severely delayed cannot be decided in our experimental setup, as the observation of developmental defects was not extended considerably beyond 6 h to avoid possible general retardation.

Discussion

We have isolated a chicken cDNA which encodes a novel bHLH transcription factor, referred to as cMeso-1. The gene is initially expressed in the epiblast and in the gastrulating mesoderm. During somitogenesis a prominent expression domain arises in the anterior portion of the segmental plate demarcating a limited region caudal to the next pair of prospective somites. This expression pattern is dynamic and appears to be established in time and space by a program that is intrinsic to the segmental plate. Loss of cMeso-1 function by an antisense approach results in severe impairment of somite formation. cMeso-1 is a protein of 287 amino acids related to the recently isolated mouse MesP1 and MesP2 genes (Sage et al., 1996, 1997). Although MesP2 has a similar expression pattern and also seems to be involved in somitogenesis, it probably is not the orthologue of cMeso-1. This is inferred from the limited sequence similarity within the bHLH domain only, while true chicken and mouse orthologues usually are extensively homologues throughout the coding region (Barnes et al., 1997). In addition, the expression patterns of cMeso-1 in chicken and MesP2 in mouse appear similar but not identical, because the mouse gene appar-

**FIG. 6.** cMeso-1 antisense RNA or oligonucleotides attenuate somitogenesis in chicken embryos. A shows numbers of somites formed within 6 h after the application of RNA dissolved in polycationic transfection reagent directly onto stage 10 embryos in ovo (for details see Materials and Methods). The open bar represents injections of SuperFect alone, dark gray columns stand for sense RNA, and light gray columns indicate antisense RNA. Figures within the bars give the number of tested embryos. B shows a time course of somite formation in explanted embryos treated with PBS alone (open bars), randomized nonsense oligonucleotides (dark gray bars), and antisense oligonucleotides (light gray bars) 2, 4, and 6 h after the start of the experiment. The accumulation of cMeso-1 transcripts after 6 h of treatment with randomized nonsense oligonucleotides is shown in C and compared to embryos treated for 3 (D) and 6 h (E) with antisense oligonucleotides. The accumulation of unrelated cFKH-1 (F) and paraxis (G) transcripts in the paraxial mesoderm and somites is unaffected by a 6-h treatment with antisense oligonucleotides. H illustrates the caudal truncation in an embryo treated with antisense oligonucleotide (left) compared to an embryo treated with nonsense oligonucleotide (right). All embryos shown are explant cultures. The significance of reduction of newly formed somites under treatment was at a P value <0.01.
cMeso1 in Somitogenesis

First cMeso-1 positive mesodermal cells migrate toward the streak and consequently accumulate medially in the paraxial mesoderm. Fates and migratory routes of primitive streak cells have been mapped extensively (Rosenquist, 1966; Schoenwolf et al., 1992; Schoenwolf and Sheard, 1990; Garcia-Martinez and Schoenwolf, 1993). According to fate maps performed by Psychos and Stern (1996) cells of the anterior primitive streak give rise to the somitic mesoderm. The regions of primitive streak which express cMeso-1 may contribute to the presomitic mesoderm and possibly to the lateral plate mesoderm, as c-Meso-1 initially is also expressed in the posterior part of the embryo. In agreement with a contribution of cMeso-1 positive cells to the paraxial mesoderm, we find cMeso-1 expression shifting from an initially more posterior position toward anterior at the late primitive streak stage when the primitive streak regresses. Interestingly, we frequently observed caudal truncations in embryos treated with antisense cMeso-1 RNA or antisense oligonucleotides. This may reflect the contribution of early cMeso-1 positive cells to the growing paraxial mesoderm. The disturbance of longitudinal growth and the formation of paraxial mesoderm in the presence of antisense oligonucleotides may well contribute to the problem of somitogenesis. However, because posterior truncations occurred to variable degrees and not with complete penetrance in antisense oligonucleotide treated embryos, while the somite segmentation defects were always observed with amazingly little variation, we believe that truncation of the body axis is a separate event and not the sole reason for the defect in somite formation. In this view, the functions of cMeso-1 expressed early in the posterior regressing domain and later in the anterior presomitic mesoderm would be distinct. However, we cannot entirely exclude overlapping cMeso-1 functions in both domains, because our antisense approach does not allow to separate cleanly both aspects.

The second expression domain of cMeso-1 is located in the anterior presomitic mesoderm initially in a stripe immediately posterior to the next prospective somites. Transcripts in this stripe seem to persist until the somites actually start to pinch off the mesenchyme. Thus, depending on the phase of the somite formation cycle in which the embryo has been fixed for analysis, one finds one or two stripes at the anterior end of the presomitic mesoderm. This cMeso-1 expression appears functionally associated with somite formation, because antisense oligonucleotides attenuate or even prevent somitogenesis. Somite formation is accompanied by cell compaction (Primmett et al., 1989), changes of the cytoskeletal organization and extracellular matrix composition (Meier, 1979; Ostrovski et al., 1988), and transformation of mesenchyme to epithelium (Ostrovski et al., 1988). It has been shown that increased cadherin and NCAM expression correlates with the increase in cell adhesiveness during maturation of the segmental plate (Bellairs et al., 1978; Duband et al., 1987). Therefore, it is conceivable that cell adhesion molecules may represent downstream target genes for cMeso-1. Fibroblast growth factor receptor 1 (FGFR-1) is upregulated in the presomitic mesoderm during maturation of somitomeres and later becomes restricted to the rostral parts of newly formed somites (Orr-Urtreger et al., 1991; Yamaguchi et al., 1992). FGFs are known to play a role in morphogenetic cell movements during gastrulation (Smith and Howard, 1992), specification of anteroposterior body axis (Slack and Tannahill, 1992) as well as cell proliferation and tissue differentiation. They may also be involved in regulating cMeso-1 gene activity or be subject to regulation by this transcription factor.

Vertebrate homologues of the Drosophila Notch/Delta gene family have been identified in Xenopus, mouse, and chick (Lardelli et al., 1994; Chitnis et al., 1995; Henrique et al., 1995; Uyttendaele et al., 1996; Jen et al., 1997) and constitute important signals for specification of cell fates. They are expressed in the neuroectoderm and in the presomitic mesoderm. Dll1 null mutant mice (Hrabe de Angelis et al., 1997) exert defects in somite formation, such as loss of craniocaudal polarity and epithelialization, which indicates that Notch/Delta signaling is essential for somitogenesis. Dll1 transcripts accumulate in the presomitic mesoderm immediately caudal to the newly formed somites overlapping with cMeso-1 expression. Preliminary results from this laboratory suggest that Delta expression is unaltered in embryos treated with antisense cMeso-1 oligonucleotides which would be consistent with cMeso-1 acting downstream of dll. The recently described MesP2 null mutant mice also show normal Delta expression but lack

**FIG. 7.** cMeso-1 expression is not influenced by anterior structures. Stage 9–10 embryos were transversely dissected at different axial levels as depicted schematically in A–D (top level). Anterior (a) and posterior (p, contrasted by blue background) pieces were cultivated separately and newly formed somites (indicated schematically in dark blue) were counted. Already formed somites and the region of the next-to-form somites at the time of the operation are shown in yellow by solid and dotted lines, respectively. The expression domain of cMeso-1 is shown in red. Middle panels schematically illustrate the formation of somites and cMeso-1 expression after the incubation period. The bottom panels show anterior and posterior pieces following separate cultivation and the expression of cMeso-1 in the posterior pieces by whole-mount in situ hybridization. Note that both somitogenesis and cMeso-1 expression proceed regardless of the presence of preformed somites (A and B) or the anterior domain of cMeso-1 in the segmental plate (C). In asymmetrically dissected embryos somitogenesis proceeds unilaterally until the same axial level is reached on both sides and somite formation and cMeso-1 expression continue in register (D). The images are representative examples of at least six embryos operated in each group of dissections.
expression of Notch-1 and -2 as well as FGFR1 implying that these genes function downstream of Mesp1 (Saga et al., 1997). Further studies will clarify the epistatic relationship of Notch/Delta and cMeso-1. Another gene with similar expression to cMeso-1 is the vertebrate fringe gene which has been isolated from mouse (Johnston et al., 1997) and chicken (Sakamoto et al., 1997). In Drosophila fringe acts upstream of Notch and is responsible for the determination of segment boundaries (Irvine and Wieschaus, 1994). The mouse fringe gene expressed in flies is functional which argues for a conserved function of the gene and possibly common pathways in both organisms (Johnston et al., 1997). It will be interesting to test the role of fringe for the expression of cMeso-1 in chicken.

First evidence for the importance of cMeso-1 in somitogenesis is provided by the loss of function experiments shown in this paper. Both antisense RNA and oligonucleotides in ovo and on explanted embryos result in specific inhibition of cMeso-1 transcripts and lead to the arrest or marked delay of somitogenesis. Thus, our results strongly support the functional involvement of cMeso-1 in somitogenesis, in particular in the segmentation process. Recently, the expression of the bHLH protein paraxis in chicken embryos has been abolished by antisense oligonucleotides resulting in a similar disruption of somite formation (Barnes et al., 1997) which is in full agreement with the phenotype of paraxis null mutant mice (Burgess et al., 1996). Since paraxis expression in the paraxial mesoderm overlaps with cMeso-1 and loss of function generates a similar phenotype, one may suspect that both genes function in one pathway or may even collaborate.

The dynamic expression of cMeso-1 in a limited domain of the anterior presomitic mesoderm raises the interesting question of how this pattern may be established. The simplest assumption would be that anterior signals emanating from the last formed somites would instruct the posteriorly adjacent mesoderm to express the gene. On the other hand, it has been shown that somitogenesis does not depend on anteroposterior signaling, as isolated segmental plate explants are fully capable of somite formation (Sandor and Amels, 1970; Packard and Jacobson, 1976; Bellairs and Veini, 1980; Sandor and Fazakas-Todea, 1980). This suggests that somites segregate according to an intrinsic mesodermal space-time program. We have tested whether cMeso-1 expression depends on the presence of anterior somites or the anterior cMeso-1 domain itself. Clearly, cMeso-1 was activated correctly in explants consisting of the posterior segmental plate without any anterior somites and without the cMeso-1 domain. The time and space of cMeso-1 gene activation appeared to be predetermined within the presomitic mesoderm together with somite formation itself (Bellairs, 1963; Packard and Jacobson 1976). Interestingly, c-hairy1, another avian bHLH gene, has recently been shown to be expressed in the presomitic mesoderm in cyclic waves which correspond to the formation time of new somites (Palmeirim et al., 1997). Like for cMeso-1, the rhythmic expression of c-hairy1 is an autonomous property of the presomitic mesoderm and not driven by the propagation of an activating signal along the anteroposterior axis. In contrast to cMeso-1 however, c-hairy1 exhibits dynamic expression which extends to the posterior end of the presomitic mesoderm in reiterated waves corresponding to each formation cycle of new somites. Thus, c-hairy1 may be part of a counting mechanism which defines the precise time point at which somitogenesis should start. Since cMeso-1 expression is not reiterated in the same cells but seems to correlate in its oscillation pattern with c-hairy1, one may speculate that it constitutes part of the link between the molecular clock and segmentation and somitogenesis. Determination of the precise epistatic relationship of both genes will help to clarify this proposition.

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REFERENCES


man: Differential expression of cNKx-2.3 and cNKx-2.5 during heart and gut development. Mech. Dev. 56, 151–163.


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