Lack of Enhancer Function in Mammals Is Unique to Oocytes and Fertilized Eggs*

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Previous studies have shown that the lack of novel coactivator activity in mouse oocytes and one-cell embryos (fertilized eggs) renders them incapable of utilizing Gal4:VP16-dependent enhancers (distal elements) but not promoters (proximal elements) in regulating transcription. This coactivator activity first appears in two- to four-cell embryos coincident with the major activation of zygotic gene expression. Here we show that whereas oocytes and fertilized eggs could utilize Sp1-dependent promoters, they could not utilize Sp1-dependent enhancers, although they showed promoter repression, which is a requirement for delineating enhancer function. In contrast, both Sp1-dependent promoters and enhancers were functional in two- to four-cell embryos. Furthermore, the same embryonic stem cell mRNA that provided the coactivator activity for Gal4: VP16-dependent enhancer function also provided Sp1-dependent enhancer function in oocytes. Therefore, the coactivator activity appears to be a requirement for general enhancer function. To determine whether the absence of enhancer function is a unique property of oocytes or a general property of other terminally differentiated cells, transcription was examined in terminally differentiated hNT neurons and their precursors, undifferentiated NT2 stem cells. The results showed that both cell types could utilize enhancers and promoters. Thus, in mammals, the lack of enhancer function appears to be unique to oocytes and fertilized eggs, suggesting that it provides a safeguard against premature activation of genes prior to zygotic gene expression during development.

Transcription factors can activate RNA polymerase II transcription mainly in the capacity of a promoter or enhancer. The promoter determines the site of transcriptional initiation, which is carried out when transcription factors bind to the DNA sequence close to and upstream from the start site. The enhancer stimulates weak promoters in a tissue-specific manner, which is carried out when transcription factors bind to their specific sequences distal to and either upstream or downstream from the start site. Fertilization of a mammalian egg by a sperm triggers a complex developmental program involving cell division, growth, and differentiation that ultimately leads to the formation of the animal. This process requires expression of genes in a tissue- and time-specific manner. One of the important mechanisms by which multicellular organisms achieve such a goal is to regulate transcription with enhancers. It is believed that enhancers stimulate weak promoters by relieving chromatin-mediated repression of promoters (1–7), a process that may involve histone acetylation (4, 5, 7–10). Enhancers can also function in stimulating DNA replication (11–13). However, it is not clear how transcriptional enhancers regulate the development of a one-cell embryo (fertilized egg) into an animal. This is primarily due to the lack of availability of sufficient number of embryos to carry out biochemical studies. Only recently techniques have been developed that allow the study of transcription and replication in mammalian oocytes, embryos as early as a fertilized egg, and even a single embryo using microinjection techniques (14–18). Such techniques have shown that DNA replication and transcription from the injected DNA are carried out in mammalian system only when appropriate eukaryotic regulatory sequences and competent cell types are present. For example, expression of genes from microinjected plasmids in mammalian oocytes and embryos requires the presence of valid promoter elements. Likewise, expression of genes from plasmids injected into morphologically arrested fertilized eggs begins only 40 h after fertilization, when the expression of genes from the embryonic genome also begins (see below). This suggests that gene expression from injected plasmids is under the same control as that from the embryonic genome. Furthermore, the injected embryos can be transplanted into the womb of a pseudopregnant female mouse, where they develop into mice (19). Therefore, results obtained with the microinjection system are not an artifact of the experimental protocol and provide an opportunity to study transcriptional regulation, including enhancer function, in the context of a living animal.

In sexually mature female mice, oocytes undergo the first meiotic reductive division to become mature unfertilized eggs. These eggs when fertilized by sperm go through the second meiotic division to produce a fertilized egg containing both a paternal and maternal haploid pronucleus. Each pronucleus undergoes DNA replication; they then fuse together during the first mitosis to generate a two-cell embryo containing one zygotic diploid nucleus per cell. The major transcription of zygotic genes begins about 40 h after fertilization, which corresponds to the two-cell stage of normal development. However, in morphologically arrested fertilized eggs, the major gene expression begins at the same time. Thus, this function appears to be regulated by a time-dependent biological clock (zygotic clock) (14, 16, 20, 21). In all of these embryos, minor transcription of genes occurs during the late one-cell stage (22–30). Prior to that stage, survival of the embryos is continuously regulated by the translation of maternally inherited mRNA present in mature and fertilized eggs (31–33). The process of translation itself is regulated mostly through post-transcriptional modification (34–38).

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Enhancer Function during Mammalian Development

EXPERIMENTAL PROCEDURES

Mouse Oocytes and Embryos—Isolation, culture and injection of CD-1 mouse oocytes and embryos were carried out as described previously (15, 17, 49). Growing oocytes were obtained from 13- to 14-day-old females and were cultured in the presence of 100 μg/ml dibutyryl cAMP to prevent meiotic maturation. Growing oocytes obtained from 2- to 3-week-old prepubertal mice are more transcriptionally active than mature oocytes obtained from older mice (50). Fertilized eggs were isolated from 8- to 10-week-old pregnant females 17 h after human chorionic gonadotrophin hormone (hCG) is injected and cultured in the presence of 4 μg/ml aphidicolin (Boehringer Mannheim) to arrest their development at the beginning of S-phase. Since the first S-phase had not yet begun at the time of isolation of fertilized eggs, aphidicolin causes them to retain their two pronuclei, male and female, throughout the experiment. Two-cell embryos were isolated by micromanipulation after hCG injection, at which time they had completed S-phase. Since two-cell embryos are isolated after they had undergone DNA replication, aphidicolin would cause them usually to cleave into four cells and then get arrested at the beginning of S-phase. In the absence of aphidicolin, most injected two-cell embryos develop into morula by 44 h. For butyrate treatment, oocytes and embryos were cultured in the presence of 2.5 mM sodium butyrate (51).

Plasmids—Various plasmid DNA containing the firefly luciferase gene (pluc) linked to the basal promoter (TATA box, pTluc), Sp1-dependent promoter (pS6(E)Tluc), or the herpes simplex virus thymidine kinase promoter (ptkluc), coupled to the Gal4-dependent enhancer (pG5TCAT) has been described (15). The expression vector for GAL4:VP16 (pSGVP) has been described previously (52), and the Gal4-dependent enhancer-expressing CAT reporter gene (pG5TCAT) has been described (15). pS6(E)Tluc and pptkluc were created by inserting the HpaI/SmaI promoter fragment from p1873 (53) containing six Sp1 sites into the HindIII sites of pTluc and ptkluc, after ligating a HindIII linker to the fragment. This places the Sp1 sites 600 bp upstream of either the basal or the tk promoter, respectively. All constructs were sequenced to identify the plasmids with the right orientation.

Plasmid DNA is prepared in 10 mM Tris-HCl (pH 7.6) and 0.25 mM EDTA (15) to the desired concentration, and ~2 pl is injected into oocytes about 2 h after collection and into either two-cell embryos between 44 and 48 h post-hCG. Oocytes and embryos that survived injection were assayed for reporter gene activity as described below.

Firefly Luciferase Assay—Firefly luciferase activity was assayed in individual embryos as described previously (15, 49). For each data point the mean value of 40–150 oocytes or embryos is used, and the variation among individual embryos is expressed as ± S.E. of the mean. Although the range of luciferase activities among individual embryos could vary as much as 1000-fold (15), the mean values obtained from several independent experiments are reproducible to within 13–25%. Moreover, the relative activity between different types of embryos and between promoters are almost reproducible, even when DNA injection is performed by different people.

Chloramphenicol Acetyltransferase (CAT) Assay—About 50 injected embryos were incubated for 44 h, harvested in 250 mM Tris (pH 8.0) at a concentration of 0.5 embryo per μl, lysed by freezing and thawing three times using dry ice/ethanol and 37 °C baths, and centrifuged at 16,000 × g for 5 min at 4 °C, and the supernatant was assayed for CAT activity as described by Sambrook et al. (54). The fraction of [14C]acet- tylichloramphenicol was measured by using a Betascope 603 (Betagen) to collect at least 100,000 emissions. These numbers were then normalized to the average total [14C]chloramphenicol present in lane of the chromatograph and expressed as cpm/embryo.

NT2 and hNT Cells and Their Characterization—NT2 cells (tetraploid) were derived from a human teratocarcinoma and have characteristics of neuronal stem cells. These cells can differentiate into neurons, glioma or astrocytic cells, and a variety of other cell types, including the hNT cells. NT2 and hNT cells are cultured in standard neural cell culture media. NT2 cells can be differentiated into functional neurons, astrocytes, and oligodendrocytes, and they are a useful model system for studying the developmental biology of the nervous system. hNT cells are a subclone of the NT2 cell line and are derived from a human teratocarcinoma. hNT cells are also a useful model system for studying the developmental biology of the nervous system. They can be differentiated into functional neurons, astrocytes, and oligodendrocytes, and they are a useful model system for studying the developmental biology of the nervous system.

By using the microinjection system, it has been found that although transcription can be regulated from the proximal promoter site in oocytes, fertilized eggs, and two-cell embryos, it cannot be regulated from the distal enhancer sites prior to the formation of a two-cell embryo (15, 16, 21, 28, 39, 40). Previously, we observed that the exogenous transcription factor Gal4:VP16 can stimulate transcription from the proximal promoter site but not from the distal enhancer site in terminally differentiated mouse oocytes or maternal and paternal pronuclei of fertilized eggs. In contrast, Gal4:VP16 was found to stimulate transcription from both promoter and enhancer sites in undifferentiated two- to four-cell mouse embryos (15, 41). A lack of enhancer activity in the proximal nucleoli of fertilized eggs was found to be caused by the absence of chromatin-mediated promoter repression, a prerequisite for delineating enhancer function (25–27, 41–46). On the other hand, the lack of enhancer activity in oocytes and the maternal pronuclei of fertilized eggs was found to be caused not by the absence of chromatin repression or functional enhancer activation protein (Gal4:VP16) but rather by the absence of enhancer-specific coactivator activity. This coactivator activity first appears in two-cell mouse embryos during development concurrent with the major onset of zygotic gene expression (ZGE)1 (15). Furthermore, the coactivator activity missing in oocytes could be provided to oocytes by microinjection of mRNA obtained from undifferentiated embryonic stem (ES) cells. This mRNA had no effect in two- to four-cell embryos presumably because of its presence in these cells at saturating levels (15).

To determine whether the lack of the enhancer-specific coactivator activity in oocytes and the maternal pronuclei of fertilized eggs was responsible for the inability of these cells to use enhancers driven by transcription factors other than those containing the specific activation domains, like Gal4:VP16, here we examined Sp1-dependent promoter and enhancer function in these cell types and control two- to four-cell embryos. Sp1 is a transcription factor that contains a glutamine-rich activation domain. We found that, just like with Gal4:VP16, oocytes and the maternal pronuclei of fertilized eggs can use Sp1-dependent promoters but not Sp1-dependent enhancers. In contrast, Sp1-dependent promoters and enhancers were both active in two- to four-cell embryos. Furthermore, the same ES cell mRNA that provided the missing coactivator activity for Gal4:VP16-dependent enhancer function also provided Sp1-dependent enhancer function in oocytes. Thus, enhancer-specific coactivator activity, which is absent prior to the formation of a two-cell embryo, is a requirement for general enhancer function. In addition, to determine whether the absence of the enhancer function is a general property of terminally differentiated cells or is only limited to oocytes, we examined both Gal4:VP16 and Sp1-dependent promoter and enhancer activity in terminally differentiated hNT neurons and their precursors, NT2 neuronal stem cells. NT2 cells can be differentiated into mature hNT neurons in the presence of retinoic acid under well-established cell culture conditions; both hNT neurons and NT2 cells have been extensively characterized (47, 48). We found that both hNT neurons and NT2 cells used Gal4:VP16 and Sp1-dependent promoters and enhancers. Thus, in mammals, the absence of the enhancer-specific coactivator, and therefore the inability to use enhancers, appears to be unique to oocytes and fertilized eggs prior to ZGE.

1 The abbreviations used are: ZGE, zygotic gene expression; ES, embryonic stem; NT2, Ntera 2; hNT, human NT; bp, base pairs; RT-PCR, reverse-transcriptase-polymerase chain reaction; GFP, green fluorescent protein; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; MBT, midblastula transition; hCG, human chorionic gonadotrophin.
To characterize these cells morphologically, they were examined by phase contrast microscopy. Biochemical characterization was done by RT-PCR and by Western blot assays to determine the pattern of expressed genes. Total mRNA was prepared from 5 × 10^6 NT2 cells and 3 × 10^6 hNT cells using the RNA isolation kit from Stratagene. First strand cDNA was prepared with the SuperScript preamplification kit from Life Technologies, Inc. Expression of neural genes was examined by RT-PCR as follows: 5 min at 95 °C followed by 40 cycles of 45 s at 95 °C, 45 s at 60 °C, and 45 s at 72 °C, followed by 7 min at 72 °C. The primers were CAACGTCTAGCTAGTC and AGGCAGAAGGCTCAGCA for Nestin, AACCTGCAAGACCGAAG and CCTTGATGACGTCTATGC for glutamate receptor, and ACCGATTGGCTATGGG and TGATTTGAGGGATCTCGCC for glyceraldehyde-3-phosphate dehydrogenase.

For Western blot analysis, total protein extracts were prepared, electrophoresed, and blotted as described (55), and the signal was revealed using the ECL kit from Amersham Pharmacia Biotech. The antibodies used were mouse anti-vimentin (Zymed Laboratories Inc.), rabbit anti-synapsin (Chemicon International), and mouse anti-actin (Amersham Pharmacia Biotech).

**Transfection and Assays of Promoter and Enhancer Activity in NT2 and hNT Cells—** Transfection of both NT2 and hNT cells was accomplished using the Stratagene MBS transfection kit (catalog number 200388) that employs the calcium phosphate precipitation method. Other methods of transfection, like electroporation and lipofection, were tried and were found to be less effective. Transfection efficiency was determined by transfection of the plasmid, pEGFPC-1 (CLONTECH), encoding green fluorescent protein (GFP) reporter gene. DNA mixtures were created by the manufacturer’s instructions with a maximum limit of 10 μg of DNA per ml of suspension mix. Cells were covered with Opti-MEM I (Life Technologies, Inc., catalog number 15985-070) with 6% MBS. One milliliter of DNA suspension was added per 100-mm plate by slowly adding drops in a circular motion. Plates were swirled once and then allowed to stand at 37 °C for 2–3 h in 5% carbon dioxide. Following incubation, the cells were washed 4–5 times with phosphate-buffered saline and fed with fresh media. For the assay of GFP expression, cells were observed under the microscope using UV light (360–400 nm) as well as visible light and were photographed. For the assay of promoter/enhancer activity, cells were harvested 48 h after the removal of the DNA suspension and assayed for reporter gene activity (luciferase, CAT, or β-galactosidase).

Luciferase assays were done on 50 μl of cell extract prepared in CEB (0.1 M sodium phosphate (pH 7.8), 1 mM dithiothreitol, and 0.1% Triton X-100) under the same conditions used for extracts of mouse oocytes and embryos as described above. Cell extracts were assayed for β-galactosidase activity using a Galacto-Light kit from Tropix. Inc., following the manufacturer’s instructions. Both assays produce a signal that is read with a Monolight 1000 machine from Analytical Luminescence Laboratory. Chloramphenicol acetyltransferase (CAT) assays also were done on 50 μl of cell extract prepared in 100 mM Tris (pH 7.6). These extracts were incubated with 4 mM acetyl coenzyme A, 0.05 μCi of [14C]chloramphenicol (Amersham Pharmacia Biotech), 0.5 mM Tris-HCl (pH 8.0) at 37 °C for 1 h, and then extracted with 900 μl of ethyl acetate and lyophilized. The pellet was dissolved in 25 μl of ethyl acetate, chromatographed on silica gel, and analyzed by autoradiography (54).

Embryonic Stem Cells— Embryonic stem (ES) cells were generated from mouse blastocystos as described (56). The ES cells were grown on 100-mm plates coated with 4 μM acetyl coenzyme A, 0.05 μCi of [14C]chloramphenicol (Amersham Pharmacia Biotech), 0.5 M Tris-HCl (pH 8.0) at 37 °C for 1 h, and then extracted with 900 μl of ethyl acetate and lyophilized. The pellet was dissolved in 25 μl of ethyl acetate, chromatographed on silica gel, and analyzed by autoradiography (54).

**RESULTS**

**Sp1 Can Activate a Promoter but Not an Enhancer Prior to Formation of a Mouse Two-cell Embryo—** To determine the relative activity of Sp1-dependent promoters and Sp1-dependent enhancers at the beginning of mouse development, plasmids containing a reporter gene linked to the appropriate regulatory sequences were injected into mouse oocytes, fertilized eggs, and two-cell embryos. The activity of each regulatory sequence was quantified by measuring the amount of luciferase activity produced several hours after injection.

The Sp1-dependent promoter consists of a tandem series of six Sp1 DNA-binding sites placed 10 base pairs upstream from the adenovirus late gene promoter (a strong TATA box) driving a firefly luciferase (luc) reporter gene (pS6LTluc) (41). The Sp1-dependent enhancer consisted of six Sp1 DNA-binding sites placed 600 bp upstream from either the adenovirus late gene promoter TATA box (pS6(luc)) or the herpes simplex virus thymidine kinase (tk) promoter (Sp1-CAT-Sp1-TATA) driving a firefly luciferase reporter gene (pS6(luc)). The tk promoter responds to stimulation by various enhancers and transactivators (41, 57–59) and thus serves as a model promoter for assessing enhancer stimulation. The level of luciferase produced in the absence of a promoter was determined by injecting pluc, a plasmid containing only the firefly luciferase gene. The level of luciferase produced by a basal promoter (TATA box) was determined by injecting pTluc.

Transcriptionally active mouse oocytes were isolated and cultured in the presence of dibutyryl cAMP to prevent meiotic maturation. Mouse fertilized eggs and two-cell embryos were isolated and cultured in the presence of aphidicolin to arrest the development of fertilized eggs as they entered S-phase and two-cell embryos at S-phase of the four-cell stage (15). Plasmid DNA was injected into the germinal vesicle of oocytes, the maternal pronucleus of fertilized eggs, and one of the two cytotelic nuclei of two-cell embryos. The amount of luciferase activity was quantified when each cell type had produced the maximum activity as described previously (15). The amount of luciferase activity observed was dependent on the amount of DNA injected, although the promoter activity in general was found to be severalfold lower in oocytes and maternal pronucleus than in two-cell embryos (15). The optimal concentration of injected plasmid DNA solution was found to be 600 μg/ml for oocytes, 150 μg/ml for fertilized eggs, and 300 μg/ml for two-cell embryos.

Although Sp1-dependent promoters were fully active in both oocytes and S-phase-arrested fertilized eggs, the Sp1-dependent enhancers were not active under the same conditions (Fig. 2). The level of basal promoter pTluc activity in oocytes was about 7-fold greater than the background pluc. The Sp1 promoter, pS6(Tluc), stimulated this basal level activity 22-fold, and the tk promoter stimulated it 51-fold. Site-specific mutations have demonstrated that the tk promoter depends on Sp1 transcription factor activity in mouse oocytes and cleavage-stage embryos (59). These results confirmed previous studies showing that oocytes contained Sp1 transcription factor activity (41, 60). However, in the present study, the Sp1-dependent enhancer stimulated the basal promoter contained in pS6(luc) only 1.7-fold, and had no effect on the tk promoter present in pS6(luc).

Similar results were obtained with fertilized eggs. In these cell types, pTluc was 13-fold more active than pluc. pS6LTluc stimulated this basal level activity 19-fold, and pTluc stimu-
moter (pS6(E)Tluc) or the tk promoter (pS6(E)tkluc). A promoterless promoter, six Sp1 sites placed 600 bp upstream of the basal promoter (ptkluc). The Sp1 enhancer plasmids carried, in addition to enhancers until formation of a two-cell embryo.

Oocytes, the maternal pronuclei of fertilized eggs, and the zygotic nuclei of two-cell embryos, but they cannot function as enhancers until formation of a two-cell embryo. CD-1 mouse oocytes were cultured in dibutyryl cAMP to prevent meiotic maturation, and two-cell embryos were cultured in aphidicolin to arrest development at the beginning of S-phase in 4-cell embryos. Plasmid DNA (2 μg/ml plasmid DNA, respectively) was injected into the germinal vesicles of oocytes or one of the two zygotic nuclei of two-cell embryos using DNA solutions containing 550, 150, and 300 μg/ml plasmid DNA, respectively. The promoter plasmids carried the firefly luciferase gene (luc) under the control of the basal TATA box promoter (pTluc), Sp1 promoter containing six Sp1 sites placed 30 bp upstream of the TATA box (pS6Tluc), or the HSV-tk promoter (ptkluc). The Sp1 enhancer plasmids carried, in addition to the promoter, six Sp1 sites placed 600 bp upstream of the basal promoter (pS6(E)/Tluc) or the tk promoter (pS6(E)/tkluc). A promoterless control (pluc) was also tested. After injection, oocytes were cultured for 20 h, and embryos were cultured for 42 h before the extracts were prepared, and luciferase activity was measured quantitatively in individual embryos or oocytes and expressed as light units (37). Each data point indicates the mean value ± S.E. for 40–60 successfully injected oocytes or embryos.

In contrast, two- to four-cell embryos utilized both Sp1-dependent promoters and Sp1-dependent enhancers. In these embryos, the tk promoter was 40-fold more active than the basal level promoter (Fig. 2), indicating the presence of functional Sp1. As expected (41), the weaker basal promoter, as in pS6/E/Tluc, was stimulated more strongly (51-fold) by the Sp1-dependent enhancer than the stronger tk promoter, as in pS6(E)/tkluc (16.5-fold). These results are similar to previous findings with Gal4:VP16-dependent promoters and enhancers in the presence of exogenously expressed Gal4:VP16 protein (15). Therefore, the inability of oocytes and fertilized eggs to use enhancers was not because of the lack of functional enhancer-specific transcription factors and whether the transcription factor was naturally present in these cells or was provided artificially by an expression vector.

Promoter Repression by Oocytes, Maternal Pronuclei of Fertilized Eggs, and Two- to Four-cell Embryos—To determine if the lack of enhancer stimulation of promoters in oocytes and the maternal pronuclei of fertilized eggs was caused by the absence of chromatin-mediated promoter repression, which is necessary to delineate enhancer function, we treated these cell types with sodium butyrate. Sodium butyrate inhibits histone deacetylase, which increases the degree of acetylation of core histones. This in turn stimulates both cellular- and plasmid-encoded mammalian genes by destabilization of chromatin repression (61, 62). Sodium butyrate treatment has also been found to destabilize chromatin repression and to cause stimulation of promoter activity in oocytes and cleavage-stage mouse embryos (15, 26, 41, 42). In the present study, we extended those findings to the Sp1 enhancer construct, pS6(E)/tkluc, and its corresponding promoter, ptkluc (Fig. 3). Sodium butyrate stimulated the tk promoter 11.7-fold in oocytes, 7-fold in maternal pronuclei of fertilized eggs, and 18.5-fold in two- to four-cell embryos. This also indicated the approximate degree of promoter repression in these cell types that, under our experimental conditions, can be potentially relieved, and therefore stimulated, by enhancers (41, 42). Previously we found that once a promoter is stimulated by an enhancer, it cannot be further stimulated by sodium butyrate; stimulation by sodium butyrate is specific for promoters unstimulated by enhancers (12, 31, 32). Accordingly, pS6(E)/tkluc, which is already stimulated by the Sp1-dependent enhancers in two- to four-cell embryos, could not be further stimulated by sodium butyrate (0.91-fold). In contrast, because the Sp1-dependent enhancer was inactive in oocytes and fertilized eggs, the pS6(E)/tkluc construct could be stimulated by sodium butyrate 12.5-fold in oocytes and 6.3-fold in fertilized eggs. Taken together, these experiments showed that, even in the presence of promoter repression and functional endogenous Sp1, oocytes and the maternal pronuclei of fertilized eggs can support only Sp1-dependent promoter activity and not Sp1-dependent enhancer activity. In contrast, two- to four-cell embryos can support both Sp1-dependent promoter and enhancer activity.

Sp1-dependent Enhancer Function Can Be Provided to Oocytes by ES Cell mRNA—Previously, we found that injection of mRNA obtained from ES cells that used enhancer function efficiently (63) could restore Gal4:VP16-dependent enhancer activity in oocytes. However, the ES cell mRNA could stimulate the enhancer function only partially. The suboptimal nature of enhancer stimulation in oocytes was also demonstrated when the mRNA level was decreased to half of its concentration by adding tRNA, which also decreased the enhancer stimulation by half. The enhancer activity could not be restored to the maximum level because a higher concentration of injected mRNA was toxic to oocytes. Thus, these experiments indicated that ES cell mRNA contained one or more factors required for Gal4:VP16-dependent enhancers (15).
To determine if the same ES cell mRNA could cause Sp1-dependent enhancers to function, about 0.5 pg of mRNA in a volume of about 2 pl was injected into the cytoplasm of oocytes, which were then kept for an hour in the 37 °C incubator to recover. The surviving oocytes were injected with either ptkluc or pS6(E)tkluc as described in Fig. 2. In some experiments, about 2 pl of 500 pg/ml ES cell mRNA was preinjected into the cytoplasm of oocytes or two-cell embryos (both blastomeres) 1 h before either of the plasmid DNAs was injected into one of the nuclei of these cells. Stimulation by Sp1-dependent enhancer in oocytes was marginal when mRNA and luciferase expression vectors were coinjected into the nuclei. ES cells were generated from mouse blastocysts and propagated on lysed primary mouse embryonic fibroblast cells as feeder layer in Dulbecco's modified Eagle's medium (Specialty Media) plus 15% heat-inactivated fetal bovine serum and in the presence of 1000 units/ml ESGRO murine leukemia inhibitory factor (Life Technologies, Inc.) to prevent them from differentiating (44). mRNA from ES cells was isolated using an RNA isolation kit from Stratagene.

Use of Gal4:VP16- and Sp1-dependent Promoters and Enhancers by NT2 Stem Cells and hNT Neurons—To determine if the lack of enhancer function in terminally differentiated oocytes caused by the absence of enhancer-specific coactivator activity was restricted to oocytes or was a general property of terminally differentiated cell types, we examined the promoter and enhancer function in undifferentiated NT2 neuronal stem cells and terminally differentiated hNT neurons. NT2 cells can be differentiated into hNT neurons in the presence of retinoic acid under tissue culture conditions (48). Before testing the transcriptional activity of these cells, we wanted to characterize them at both the cellular and molecular levels (Fig. 5). The morphological features of these cells were examined by phase-contrast microscopy. As expected, cell division arrested hNT neurons (Fig. 5B), and dividing NT2 cells (Fig. 5A) showed characteristic morphology. More than 99% of the NT2 cells can be converted into hNT neurons by this procedure. To determine the expression of cell type-specific markers in these cells at the

Fig. 4. Enhancers can be activated in mouse oocytes by preinjection of ES cell mRNA. Oocytes or two-cell embryos were injected with either ptkluc or pS6(E)tkluc as described in Fig. 2. In some experiments, about 2 pl of 500 pg/ml ES cell mRNA was preinjected into the cytoplasm of oocytes or two-cell embryos (both blastomeres) 1 h before either of the plasmid DNAs was injected into one of the nuclei of these cells. Stimulation by Sp1-dependent enhancer in oocytes was marginal when mRNA and luciferase expression vectors were coinjected into the nuclei. ES cells were generated from mouse blastocysts and propagated on lysed primary mouse embryonic fibroblast cells as feeder layer in Dulbecco's modified Eagle's medium (Specialty Media) plus 15% heat-inactivated fetal bovine serum and in the presence of 1000 units/ml ESGRO murine leukemia inhibitory factor (Life Technologies, Inc.) to prevent them from differentiating (44). mRNA from ES cells was isolated using an RNA isolation kit from Stratagene.

FIG. 3. Butyrate can stimulate promoters in oocytes, the maternal pronuclei of fertilized eggs and the zygotic nuclei of two-cell embryos, whereas Sp1-dependent enhancers can stimulate promoters only in two-cell embryos. The injection and assay of promoter and enhancer activities were carried out as described in Fig. 2, except that some of the oocytes and embryos were cultured in the presence of 2.5 mm sodium butyrate. The tk promoter can be stimulated by butyrate (+/− butyrate) 11.7-fold in oocytes, 7-fold in fertilized eggs, and 18.5-fold in two-cell embryos. However, the tk promoter cannot be stimulated by Sp1 enhancer (pS6(E)tktk) in oocytes and fertilized eggs but can be stimulated 19-fold in two-cell embryos. The unstimulated tk promoter present in the enhancer construct pS6(E)tk can be stimulated 12.5-fold in oocytes and 6.3-fold in fertilized eggs. Since the Sp1-enhancer already stimulates the tk promoter in two-cell embryos, butyrate cannot stimulate it any further. The horizontal line across the figure in the bottom panel denotes the stimulation of 1-fold.
To determine further the expression of specific markers in these cells at the protein level, cell extracts were subjected to Western blot analysis using anti-vimentin, a stem cell marker, and anti-synapsin, a neuronal marker (48). Anti-actin was used as a control because the housekeeping actin gene is expressed in both cell types. As shown in Fig. 5D, vimentin was detected in NT2 cells but not in hNT neurons, whereas synapsin was detected in hNT neurons but not in NT2 cells. Taken together, these experiments showed that NT2 cells exhibited the characteristics of neuronal stem cells, whereas hNT neurons exhibited those of terminally differentiated neurons.

The transcriptional properties of NT2 cells and hNT neurons were examined by transient transfection assay. Usually, terminally differentiated neurons are not amenable to transfection with high efficiency. In the present study, transfection methods employing electroporation or lipofection did not yield measurable transfected reporter gene activity. A calcium phosphate transfection method using a commercially available kit was found to be more useful. Transfection of the plasmid, pEGFP-C1, encoding the green fluorescent protein (GFP), was carried out to determine transfection efficiency. Under the experiment conditions, 40% of NT2 cells and about 1% of hNT neurons expressed GFP. Although more than 99% of the hNT population consisted of neurons expressing neurite outgrowths, and the hNT neurons did not express stem cell markers in the RT-PCR assay (indicating that, if not all, of the cells in hNT population were postmitotic neurons), we wanted to ascertain that the reporter gene activity obtained from transfected hNT neurons was actually generated by differentiated neurons. For this purpose, cells were photographed under both ultraviolet light (360 to 400 nm) and visible light after transfection with pEGFP-C1. All transfected cells expressing GFP were found to be differentiated postmitotic neurons (Fig. 5, E and F). Thus, these experiments indicated that the expression of transfected reporter gene activity in the hNT neurons was actually contributed by differentiated neurons, not by possible contamination of NT2 stem cells.

Under these experiment conditions, the promoter and enhancer activity was then quantitatively evaluated in NT2 cells and hNT neurons by their ability to express the reporter gene. Before examining the Sp1- and Gal4-VP16-dependent enhancer activity in these cells, we wanted to ensure that, under the conditions of the experiment, these cells contained functionally active Sp1 and Gal4-VP16. The activity of endogenous Sp1 was tested by comparing the promoter activity of the basal promoter, pLUC, and the Sp1-dependent promoter, pS6LUC. Similarly, the activity of exogenous Gal4-VP16 was tested by comparing the activity of Gal4-VP16-dependent promoter, pG5TCAT, in the absence and presence of pSGVP, an expression vector for Gal4-VP16 protein (41). The plasmid pRSV-β-gal encoding the β-galactosidase reporter gene was cotransfected in each of the individual transfection assays as an internal standard. The total amount of plasmid transfected was kept constant in each experiment by adding the vector plasmid pBR322. The cell extract from each individual experiment was assayed for luciferase and β-galactosidase activity. Each luciferase activity was then normalized to the β-galactosidase activity. The mean values for two or more such experiments are plotted in Figs. 6 (NT2 cells) and 7 (hNT neurons).

The Sp1-dependent promoter, as compared with the basal promoter, produced 15- and 7-fold higher promoter activity in NT2 cells and hNT neurons, respectively, indicating that, under these conditions, endogenous Sp1 can function from the proximal promoter site in both of these cells. Likewise, the Gal4-VP16-dependent promoter alone was inactive in both NT2
cells and hNT neurons, but cotransfection with pSGVP stimulated the promoter activity 22-fold in NT2 cells and 9-fold in hNT neurons, again indicating that, under these conditions, these cells expressed functional Gal4:VP16 protein. The Sp1- and Gal4:VP16-dependent constructs were cotransfected with an expression vector, pSGVP. pTluc was used to monitor the basal promoter activity. The plasmid pRSV-β-gal expressing β-galactosidase as a reporter gene was cotransfected as an internal control in each transfection experiment. The total amount of the transfected plasmid DNA was maintained at 10 μg per transfection. Cells were harvested 48 h after transfection, and the cell extracts were assayed for luciferase or CAT. Luciferase or β-galactosidase activities were calculated from the values (pS6(E)tkluc/ptkluc) and (pG9(E)tkluc/ptkluc), respectively.

### DISCUSSION

We drew two conclusions from our present studies of gene expression in mammalian systems, namely terminally differentiated oocytes, fertilized eggs, and two- to four-cell embryos on one hand and terminally differentiated hNT neurons and their precursor, NT2 stem cells on the other. First, the enhancer-specific coactivator activity is required for enhancers driven by both Gal4:VP16 (acidic activation domain) and Sp1 (glutamine-rich activation domain), suggesting that the coactivator activity is a requirement for general enhancer function. Second, the lack of coactivator activity, and therefore enhancer function, is unique to oocytes and fertilized eggs. This coactivator activity first appears in two- to four-cell embryos, suggesting that this mechanism provides a safeguard against premature activation of zygotic genes prior to ZGE. Various aspects of enhancer function during mouse embryonic development are discussed below.

**Lack of Enhancer-specific Coactivator Activity in Terminally Differentiated Mouse Oocytes**—Multicellular organisms, as opposed to their unicellular counterparts, face a unique problem in carrying out life-sustaining functions. Whereas in unicellular organisms the same cell performs all the necessary func-
tions, in multicellular organisms there is a division of labor: specific cell types carry out specific functions in a spatial and temporal manner. During the mammalian life cycle, multipotent progenitor stem cells undergo differentiation at various levels, eventually giving rise to specific cell types. Whereas the stem cells maintain the potential for cell division, giving rise to either their own kind or a differentiated form, the terminally differentiated cells lose such potential. In fact, oocytes and neurons are two examples of terminally differentiated cell types that lose the ability to undergo division completely. Although these non-dividing cells are presumed to perform restricted tasks, the strategies they adopt to regulate transcription is unknown. In the present study, we used differentiated oocytes and undifferentiated two- to four-cell embryos to examine such strategies and to compare them with undifferentiated neuronal NT2 stem cells and differentiated hNT neurons.

Previously, we found that transcription in mouse oocytes is regulated by an exogenously added Gal4:VP16-dependent promoter activity but not Gal4:VP16-dependent enhancer activity. In contrast, both promoter and enhancer activity were observed in two- to four-cell embryos. Enhancers are believed to stimulate promoters by relieving chromatin-mediated repression. However, the lack of enhancer-mediated transcription in oocytes, as compared with two- to four-cell embryos, was found to be caused not by the absence of promoter repression or functional enhancer activation protein. Accordingly, the efficiency of chromatin assembly on the microinjected DNA in mouse oocytes and two-cell embryos by assaying the degree of super-helicity was found to be equal in these cell types (−70%) (39, 65). Furthermore, this degree of chromosome assembly on the microinjected plasmid DNA was found to correlate directly with the level of repression of promoters present on these plasmids. The repression could be relieved in both oocytes and two- to four-cell embryos by histone deacetylase inhibitors like sodium butyrate or trichostatin A (41, 42, 51, 66). In contrast, the lack of enhancer function was found to be caused by the absence of an enhancer-specific coactivator activity. The missing coactivator activity could be supplied to oocytes by micro-injection of mRNA from undifferentiated ES cells (15). In the present study, we examined the question of whether the coactivator activity is also required for enhancers regulated by transcription factors other than those containing an acidic activation domain like Gal4:VP16. This was determined by testing the endogenously present Sp1-dependent promoter and enhancer activity in oocytes. We found that oocytes show Sp1-dependent promoter activity but not Sp1-dependent enhancer activity, suggesting that the coactivator activity is a general requirement for enhancers driven by various classes of transcription factors.

To determine if the lack of enhancer-mediated transcription is a general phenomenon of terminal differentiation, we further examined promoter- and enhancer-mediated transcription in NT2 stem cells and terminally differentiated hNT neurons. We found that both cell types are capable of promoter- and enhancer-mediated transcription. Therefore, neurons, but not oocytes, utilize enhancer-mediated transcription and provide evidence that different terminally differentiated cell types can regulate transcription using distinct strategies.

Lack of Enhancer-specific Coactivator Activity in Fertilized Mouse Eggs Prior to ZGE—As mentioned above, the major expression of zygotic genes takes place about 40 h after fertilization and is regulated by a time-dependent zygotic clock. This mechanism presumably involves the destruction of an inhibitor or production of a functional activator of general transcription. What is the importance of the zygotic clock that delays the onset of embryonic transcription until a defined time after fertilization? The paternal genome in sperm comes with protamines, whereas the maternal genome in eggs comes with a normal complement of core histones (67, 68). After fertilization, their genomes undergo chromatin remodeling to establish the zygotic genome at the two-cell stage. In the male pronuclei, this process of remodeling might generate DNA that is not complexed with either histones or protamines (69) or might produce a chromatin state that exposes promoters to transcription factors. Thus, the zygotic clock may provide a mechanism to ensure no spurious transcription occurs during the remodeling period. On the other hand, after zygotic remodeling, the chromatin-mediated repression of most promoters in two-cell embryos may provide a mechanism for enhancer-mediated tissue-specific transcription of genes during development and growth. Delaying expression of the enhancer-specific coactivator prior to ZGE may provide an additional mechanism for preventing inappropriate transcription of genes during this critical period of development.

The same mechanisms that regulate the beginning of ZGE during mouse embryonic development also seem to occur in other animals. In other mammals, transcription is delayed until the two-cell or 16-cell stage, presumably by the same zygotic clock mechanism. For example, ZGE begins at the two-cell stage in hamsters, the four-cell stage in pigs, the four- to eight-cell stage in humans, and the eight- to 16-cell stage in sheep, rabbits, and cows (21, 70, 71). Whether enhancer-specific coactivator activity appears during the two-cell stage of embryonic development in these mammals or is delayed until the same stage when zygotic transcription begins remains to be seen.

Special Features of DNA Replication and Transcription at the Beginning of Mammalian Development—Our present knowledge of the principles that regulate the early development of vertebrate embryos comes mainly from the Xenopus system (7, 72). During the last few years, elegant experiments have uncovered clues in diverse areas from how DNA replication and chromatin structure affects gene expression to the role of post-transcriptional modification on the expression of maternal mRNAs (10, 73, 74). Although similar in many respects, the mammalian system, as exemplified by the mouse development, appears to be mechanistically different from Xenopus in some other aspects. For example, In Xenopus oocytes, the majority of RNA polymerase II transcripts are initiated at incorrect sites (75), and transcriptional regulatory components that are required by cells at later stages in development are dispensable in oocytes (76, 77). Even more striking is the fact that although bidirectional DNA replication is initiated at specific sites in the chromosomes of differentiated mammalian cells (11, 12, 78), virtually any DNA injected into non-mammalian eggs undergoes semiconservative replication, and early embryos of amphibians and flies recognize at least 5 times more initiation sites than do differentiated cells from the same animals (79). Earlier studies showed that the activity of promoter/enhancer sequences injected into Xenopus eggs is generally delayed until the midblastula transition (MBT) (80, 81), although they appear to exhibit a low but constant rate of gene expression per cell prior to the MBT (82). Thus, the MBT in Xenopus development appeared to be equivalent to ZGE in the mouse. However, more recent studies showed that hormone-dependent transcriptional activation can be observed in Xenopus oocytes (83, 84). Another such difference between Xenopus and mouse is that histone deacetylase inhibitors do not cause accumulation of hyperacetylated histone H4 until the MBT, whereas in the mouse, histone H4 acetylation patterns are observed both in one-cell and two-cell embryos (44, 66). One possible reason for the difference in DNA replication and tran-
scription at the beginning of development between *Xenopus* and mouse is that *Xenopus* oocytes contain exceptionally high concentrations of maternally inherited mRNA and proteins that permit rapid cell cleavage in the absence of transcription (85). For example, a fertilized *Xenopus* egg undergoes 11 cleavages to produce ~4000 cells in 6 h to reach the MBT, whereas a fertilized mouse egg undergoes only one cleavage event in the first 24 h to reach the two-cell stage. Thus, it appears that the transcriptional regulations observed prior to the MBT stage of frog development do not necessarily correspond to those found prior to the two-cell stage of mouse development.

**Role of DNA Replication in Enhancer Function**—In this paper, we observe enhancer-mediated stimulation of transcription to be present in all mammalian cell types, except oocytes and fertilized eggs. It has been previously shown that the absence of enhancer function in mouse oocytes was neither due to the absence of enhancer activation protein nor due to chromatin-mediated promoter repression. Rather, the lack of enhancer function in oocytes was due to the absence of an enhancer-specific coactivator activity, and this activity can be supplied to oocytes by coinjection of embryonic stem cell mRNA (15). However, experiments described in those studies and in the present study utilized non-replicating microinjected plasmid DNA as a transcriptional template in various mammalian cell types. So, the question arises whether the use of non-replicative plasmid DNA, as opposed to replicative DNA, could explain the lack of enhancer function in mammalian oocytes and fertilized eggs, because the non-replicative DNA may have different chromatin assembly as compared with replicative DNA. It has been found in *Xenopus* oocytes that DNA replication-coupled chromatin assembly is required for the efficient repression of basal transcription (86, 87). However, these authors showed that enhancer-mediated transcription through the transcription factor Gal4:VP16 is independent of DNA replication. Since promoter repression is stronger in DNA replication-coupled templates, Gal4:VP16 could stimulate the promoter to a higher extent from these templates than non-replicating templates. However, the final level of promoter stimulation by Gal4:VP16 from both templates was approximately the same (86). Since we are studying here the overall stimulation of transcription by enhancers, irrespective of the level of repression of the basal promoter, the replication status of the template should not affect the results. This view is supported by the fact that the enhancer function in mouse embryos was observed from both replicating and non-replicating DNA templates when tested in a permissive cell type (see below).

Mouse oocytes are arrested in meiosis and therefore do not replicate their own DNA. Accordingly, it has been found that mouse oocytes do not replicate plasmid DNA injected into their nucleus, even if the injected DNA contains a viral origin and is provided with the appropriate viral replication protein (65, 88). This is in direct contrast to *Xenopus* oocytes (see above). However, mouse oocytes do express some of their own genes, and they also can express genes encoded by plasmids if an appropriate promoter is present (38, 89, 90, 106). Mouse fertilized eggs do replicate their own DNA. Accordingly, they were found to replicate microinjected plasmid DNA when the DNA template contained a polyoma virus origin “core” sequence in cis and was provided with the polyoma virus replication protein, large T antigen. Even from these replicating DNA templates, enhancer function was not observed in mouse fertilized eggs (91). Thus, utilization of a replicating plasmid DNA, as opposed to non-replicating DNA, cannot be used to explain the lack of enhancer function in mouse fertilized eggs. Furthermore, mouse two-cell embryos that replicate their own DNA also replicate microinjected DNA under conditions as described for fertilized eggs above. However, in these embryos, enhancer function was observed from both replicating as well as non-replicating plasmid DNA (39, 91). Thus, the replication status of the DNA template appears not to affect the final level of promoter stimulation in mouse embryos. Whether the chromatin-mediated repression of the basal promoter is more efficient on a replicating DNA template as compared with a non-replicating DNA template in mouse two-cell embryos is not yet known.

**The Nature of Enhancer-specific Coactivator Activity**—The coactivator activity discussed here is mandatory for enhancer function but dispensable for promoter activity. Because both Sp1 and Gal4:VP16 can function from proximal promoter sites but not distal enhancer sites in oocytes and fertilized eggs, proximal and distal interactions that regulate transcription appear to involve distinct interactions engaging distinct coactivators. This hypothesis is supported by the fact that activation domains of some transcription factors have been found to function only from proximal sites, whereas others have been found to function from both proximal and distal sites (92–95). Furthermore, the C-terminal domain of RNA polymerase II has been found to be required for distal interactions, whereas the N-terminal domain of TATA-binding protein is required for proximal interactions (96–98). Such distinct interactions were also observed during our previous studies of tk promoter activity during mouse development (59). Stimulation of the promoter by either an enhancer or transactivator operated through the TATA box of the promoter in differentiated cells but switched to the distal Sp1 site of the promoter in undifferentiated cleavage-stage embryos and ES cells.

Enhancer-specific interactions have also been found in the regulation of the T cell receptor α-chain gene by its 3’ enhancer in an *in vitro* system (99, 100), where DNA topology and an architectural factor (HMG I/Y) are critical. General coactivators like PC4 (p15) that are not required for RNA polymerase II binding but are obligatory for activated transcription from a promoter site have been discovered (101, 102). Whether such coactivators can also stimulate transcription from enhancer sites is not known. The molecule(s) responsible for the enhancer activity has not yet been identified. Whether this activity is brought about by a single molecule or a family of molecules is also not known. Presumably the coactivator activity mediates protein-protein interaction between factors bound at the enhancer site and the transcription complex bound at the promoter site. Whether this interaction involves DNA topology, DNA tracking, and/or a DNA looping mechanism is not yet clear. However, because the chromatin structure and enhancer function are intimately connected, the coactivator activity very likely acts by remodeling the chromatin structure. Recently, several factors such as the SWI/SNF complex, RSC (STH1), NURF (ISWI), MOT1, ACF, FACT, and others that modulate the chromatin structure either directly or indirectly have been identified (103–107). The actual chromatin destabilization process may involve histone acetylation or other modifications (4, 5, 7–9). Whether enhancer-specific coactivator activity is represented by any or all of these complexes is not clear. However, our preliminary experiments showed that a fraction of the nuclear extract from HeLa cells can restore enhancer activity in oocytes.² The identification of the protein or the protein complex that provides the enhancer-specific coactivator activity would further our understanding of transcriptional regulation through enhancers.

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² L. Rastelli, Z. Zhao, and S. Majumder, unpublished observations.
