Expression and Function of Bicarbonate/Chloride Exchangers in the Preimplantation Mouse Embryo*

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Bicarbonate/chloride (HCO₃⁻/Cl⁻) exchangers regulate intracellular pH in the alkaline range. Previously, it has been shown that mouse embryos at the two-cell stage exhibit this activity, but that the otherwise ubiquitous mechanisms for regulating intracellular pH in the acid-to-neutral range are undetectable. We have examined mouse embryos during preimplantation development (one-cell zygote through blastocyst) to determine whether HCO₃⁻/Cl⁻ exchange activity exists at all stages, whether it is necessary for preimplantation development, and whether messenger RNAs from the known HCO₃⁻/Cl⁻ exchanger genes are expressed. We have found that all stages of preimplantation embryo have detectable HCO₃⁻/Cl⁻ exchange activity. In addition, inhibition of this activity with the stilbene anion exchange inhibitor DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid) disrupts intracellular pH homeostasis and markedly inhibits embryo development from the two-cell stage to blastocysts in culture under conditions of moderately high external pH. Finally, mRNA encoding two members of the band 3-related AE anion exchanger gene family are expressed in preimplantation embryos.

The two-cell stage mouse embryo has been shown to exhibit HCO₃⁻/Cl⁻ exchange activity which mediates recovery from intracellular alkalosis (1). Biochemically, this activity is much like HCO₃⁻/Cl⁻ exchange activities found in many cultured mammalian cells: it is inhibitable by the stilbene drug DIDS,¹ is active above about pH 7.2 and has a Kₘ for external Cl⁻ in the millimolar range (1). Surprisingly, there was no detectable corresponding activity of mechanisms to correct deviations of pH in the acid direction, such as the otherwise ubiquitous Na⁺/H⁺ antiporter (2) or the Na⁺,HCO₃⁻/Cl⁻ exchanger (3). Similarly, the unfertilized mouse egg has been reported to lack Na⁺/H⁺ antiport activity (4). Thus, HCO₃⁻/Cl⁻ exchange appears to be the sole pH regulatory mechanism in the early embryo (at least at the two-cell stage).

Three genes encoding HCO₃⁻/Cl⁻ exchangers have been identified in mammals (5). All are related and are homologs of the erythroid anion exchanger, band 3, which functions as both a HCO₃⁻/Cl⁻ exchanger and a membrane anchor of the cytoskeleton in erythrocytes. These homologs are designated AE1, AE2, and AE3 ("AE" for "anion exchanger"). The AE1 gene encodes at least two alternate polypeptides, erythroid band 3 (6), and an N terminally truncated kidney-specific form (7, 8) apparently active in renal acid secretion (9). The AE2 gene encodes at least one polypeptide, which is widely distributed among various tissues and cultured cell lines (10, 11) where it mediates pH₁ regulation (12, 13), and/or volume regulation (14). The AE3 gene encodes at least two alternate transmembrane polypeptides with differing N termini. One was first cloned from brain and is termed the "brain" isoform (11, 15, 16), while the other was cloned from heart and is termed the "cardiac" isoform (16, 17). In addition, alternatively spliced mRNA encoding a polypeptide lacking the transmembrane domain has been described (18). It is not yet known if all HCO₃⁻/Cl⁻ exchangers are members of the AE family, or if unrelated proteins also serve this function. However, the physiological properties of HCO₃⁻/Cl⁻ exchange in all those cell types where it has been examined resemble those of the proteins of the AE family.

pH₁ regulation by HCO₃⁻/Cl⁻ exchange has similar properties in various nucleated cell types and also when mediated by heterologously expressed AE polypeptides: HCO₃⁻/Cl⁻ exchange is activated above a threshold pH₁, which is usually around 7.1–7.3 (1, 19, 20–22). This threshold "set point" can be altered by metabolic alteration of the cell (12, 22). The activity is inhibited by stilbene drugs such as DIDS, requires HCO₃⁻ in the cell, and requires an inwardly directed Cl⁻ gradient. HCO₃⁻ and Cl⁻ compete for the same transport sites, and the apparent Kₘ for both anions is similar (generally in the range of 1–10 mM; 1, 12, 26).

While HCO₃⁻/Cl⁻ exchange activity has been demonstrated in the two-cell mouse embryo, neither the molecular basis of this HCO₃⁻/Cl⁻ exchange, nor a requirement for HCO₃⁻/Cl⁻ exchange activity in early development has been described. In addition, there has been no information available on the presence or absence of HCO₃⁻/Cl⁻ exchange activity in other stages of preimplantation embryo. The studies presented here address these questions.
Materials and Methods

Embryos—Embryos were obtained from superovulated (intraperitoneal injections of 5 IU PMSG followed 48 h later by 5 IU HCG, Sigma) CF1 female mice mated with BDF males (Charles River, Canada). One-cell stage embryos, two-cell stage embryos, morulae, and blastocysts were obtained 22–24, 44–48, 70–72, and 94–96 h post-hCG, respectively. One- and two-cell stage embryos were obtained by flushing excised oviducts with KFHM medium (described below), morulae were obtained by similarly flushing excised oviduct-uterus complexes, and blastocysts were obtained by flushing excised oviduct-uterine horn complexes or uterine horns alone. The flushed embryos were collected and washed three times in KFMH using mouth-operated, flame-pulled glass pipettes.

Media—KSMO culture medium (24) contains (in mM except as noted) 95 NaCl, 2.5 KCl, 0.35 KH₂PO₄, 0.2 MgSO₄, 10 sodium lactate, 10 HEPES (pH 7.4 at 37°C), was used for obtaining and handling the embryos.

Detection of Functional HCO₃⁻/Cl⁻ Exchangers in Embryos—HCO₃⁻/Cl⁻ exchange can be detected by monitoring pH; during replacement of the external medium with one which lacks Cl⁻ (3, 19, 26). The subsequent efflux of Cl⁻ through any HCO₃⁻/Cl⁻ exchanger(s) present obliges an import of HCO₃⁻ into the cell, increasing pH. The exchanger runs in the reverse direction to that required by its usual physiological role but still effects HCO₃⁻/Cl⁻ exchange. Activity is confirmed by showing that the rise is abolished by DIDS but independent of external Na⁺.

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SNARF-1-loaded embryos were placed into a temperature- and atmosphere-controlled chamber (37°C and 5% CO₂; Biophysics Inc., Baltimore, MD) fitted with a perfusion apparatus (solution changed in <30 s). After monitoring pH, for 10 min, the medium (KSOM with 9 mM sodium lactate replaced by NaCl) was replaced by a medium that was identical except that Cl⁻ was replaced by gluconate and sulfate (Cl⁻⁻ = 0, nominally); 100 µM DIDS was used to assess inhibition, and Na⁺- and Cl⁻⁻-free medium was used to assess Na⁺ dependence (NaCl, sodium lactate, and sodium pyruvate replaced by isomotic sucrose, remainder of Cl⁻ replaced by gluconate, and HCO₃⁻ supplied by choline HCO₃⁻). Embryos at the one-cell, two-cell, morula, and blastocyst stages were used. Blastocysts were mechanically collapsed by passage through a narrow-bore pipette to allow DIDS access to the blastocoele cavity (27). Three measurements, with and without DIDS, and two measurements without Na⁺, were carried out at each stage (8–20 embryos/measurement group). The data, expressed as the mean pH, of the group of embryos at each time point, were analyzed by determining the initial rate of pH increase after Cl⁻ removal, and by determining the maximum net change in pH. Initial rate of pH increase was determined by a linear regression performed on the linear portion of the increase, constituting the first seven (one-cell stage) or 10 (other stages) data points (taken at 30-s intervals). The net increase was obtained between the baseline pH before Cl⁻ replacement or Cl⁻ removal, and the peak or plateau pH following Cl⁻ removal (both averaged over 5 min). The statistical analysis was performed to determine whether the initial rate of increase and/or net increase were significantly different in the presence or absence of DIDS; this was done by t test (assuming unequal variances) at each stage. The data were also analyzed to determine if the initial rate of increase or net increase in pH, in the presence or absence of DIDS, was significantly different between the one- and two-cell stages, and between the morula and blastocyst stages.
Polymerase Chain Reaction (PCR) Determination of Anion Exchanger mRNA in Embryos—To detect cDNAs reverse transcribed from anion exchanger mRNAs in embryos, 30 cycles of PCR were performed as described below. In addition, for increased specificity and increased sensitivity in detecting the small amounts of mRNA produced by a few embryonic anion exchanger genes, 30–60 cycles of PCR protocol was used. In which case, the PCR cycles of the 30-cycle PCR were diluted 100-fold, and then a second, 20-cycle round of PCR was performed using the same 5′ primer, and a 3′ primer internal to the first one. Primers, close to the 3′ end of the translated sequence, were designed. The PCR primers sequences were used for AE1, AE2, and AE3 (Oligo 4.1, National Biosciences, Plymouth, MN) and synthesized (Beckman Oligio 1000). The sources of the mouse anion exchanger cDNA sequences used were for AE1, AE labels, for AE2, and for AE3, 15. For the PCR primers sequences were used. The Perkin Elmer Cetus PCR kit with Taq DNA polymerase was used with 1 μl of embryo cDNA (equivalent to 2.5 embryos) or diluted 30-cycle product, in 20 μl total volume. The product sizes from cDNA were for the 5′, 3′ pair (30-cycle PCR), AE1, 303; AE2, 280; AE3, 449; for the semi-nested PCR (5′, 3′-internal pair), AE1, 237; AE2, 210; AE3, 301. These primers flank at least one intron in each genomic sequence so that any product due to contaminating genomic DNA would be identifiably larger than that arising from cDNA, and embryo samples in which reverse transcriptase was omitted were all negative (not shown). Thus, any detected bands must arise from mRNA. To control for the possibility that our semi-nested PCR protocol was too sensitive and was detecting "leaky transcription" (see "Discussion"), we used cDNA derived from tissues known not to express significant levels of a given AE message. Each negative control tissue was run in parallel with a positive tissue. For AE1, stomach was used as a negative control, and spleen as positive (11); for AE3, spleen was used as negative and heart as positive (11). Unfortunately, there is no tissue which has been definitively shown to be negative for AE2, and thus no obvious negative control (10, 11); stomach, brain, heart, and kidney were all positive. Neither of the negative control tissues showed any detectable band of the expected size, while all positive controls were clearly positive (data not shown). Thus, any mRNAs detected by our PCR methods are unlikely to result from over-amplification and detection of non-physiological ("leaky") transcription.

Restriction Digest Confirmation of PCR Products—The identities of the semi-nested PCR products were confirmed by restriction analysis. The predicted AE1 product should yield BglI fragments of 137 and 100; the predicted AE2 should yield Rsal fragments of 146 and 64; the predicted AE3 product should yield HindIII fragments of 170 and 131. To obtain enough PCR product for restriction enzyme digestion, semi-nested PCR reactions were carried out, as described above, except that the second round (using the 5′ and 3′-internal primers) was carried out for 30 rather than 20 cycles and was done at a 5-fold larger scale (100 μl total). The products of the semi-nested PCR reactions were then purified from an agarose gel (3:1 Nusieve:Seakem, FMC) and visualized with ethidium bromide. The predicted product sizes from cDNA were for the 5′, 3′ pair (30-cycle PCR), AE1, 303; AE2, 280; AE3, 449; for the semi-nested PCR (5′, 3′-internal pair), AE1, 237; AE2, 210; AE3, 301. These primers flank at least one intron in each genomic sequence so that any product due to contaminating genomic DNA would be identifiably larger than that arising from cDNA, and embryo samples in which reverse transcriptase was omitted were all negative (not shown).

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greatly inhibited the development of embryos, with fewer than 20% reaching the blastocyst stage at 0.4 and 0.8% as compared to the 70–80% in the absence of DIDS (Fig. 1). To show that the effect of DIDS was significant, and that it depended on an interaction with CO₂, the full data set was analyzed by exact logistic regression analysis. The regression model assumed dependence on DIDS and an interaction between CO₂ and DIDS (model: Logit(proportion of blastocysts) = β₀[DIDS] + β₁[DIDS]×[CO₂] + β₂; a very good fit was demonstrated by the Hosmer-Lemeshow test, p = 0.88, and Deviance test, p = 0.29). This model was chosen after models in which a [CO₂] term had been included failed to give an adequate fit by the Hosmer-Lemeshow test, and also failed to converge, indicating that dependence on [CO₂] alone does not contribute significantly to the overall fit. This analysis showed that both the effect of DIDS and the interaction between DIDS and CO₂ were highly significant (both β₁ and β₂ were highly significantly different from zero, p < 10⁻⁴). Therefore, DIDS, but only in conjunction with lowered CO₂ level, significantly decreases overall development.

Intracellular pH (pHᵢ) of Two-cell Embryos under Culture Conditions—To see if the inhibition of development which occurs when low [CO₂] (high external pH) and DIDS are combined was secondary to an increase in intracellular pH, pHᵢ was measured under the same conditions in which embryos were cultured. The presence of the HCO₃⁻/Cl⁻ exchange inhibitor DIDS resulted in significantly elevated pHᵢ, relative to control (Fig. 1). The elevation was much more pronounced at lower CO₂ levels. The vertical axes show the measured fluorescence emission intensity ratio (left) and the calculated pHᵢ (right). The small filled symbols represent the mean pHᵢ from four to six pooled replicates (36–74 embryos); the box plots superimposed at each point represent the population spread of the data: the center line is the median, while the upper and lower bounds of the box are the 75th and 25th percentiles, respectively, and the whiskers show the 10th and 90th percentiles. See text for experimental details and data analysis.

Fig. 2. Intracellular pH of two-cell embryos after 3-5 h in culture as a function of CO₂ concentration with and without the HCO₃⁻/Cl⁻ exchange inhibitor DIDS. Using the intracellular pH-sensitive fluorophore SNARF-1, the fluorescence emission intensity ratio (640–600 nm) and hence intracellular pH (pHᵢ) was measured under the same conditions in which embryos were cultured. The presence of the HCO₃⁻/Cl⁻ exchange inhibitor DIDS resulted in significantly elevated pHᵢ, relative to control (Fig. 1). The elevation was much more pronounced at lower CO₂ levels. The vertical axes show the measured fluorescence emission intensity ratio (left) and the calculated pHᵢ (right). The small filled symbols represent the mean pHᵢ from four to six pooled replicates (36–74 embryos); the box plots superimposed at each point represent the population spread of the data: the center line is the median, while the upper and lower bounds of the box are the 75th and 25th percentiles, respectively, and the whiskers show the 10th and 90th percentiles. See text for experimental details and data analysis.

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Analyzing these data was performed in two steps. First, the effect of varying CO₂ alone, at constant [DIDS], was tested by ANOVA, treating the [DIDS] = 0 and [DIDS] = 100 uM groups separately. For each group, it was found that varying CO₂ had a highly significant effect on pHᵢ (both p < 10⁻⁴). However, the effect was small in the absence of DIDS, and, occurred only at 0.8% CO₂, but not at 1.7 or 5% CO₂ (Fig. 2). The second analysis tested the significance of the effect of DIDS and was performed on the entire data set. For this purpose, the data set can be considered to be stratified by CO₂ level, and the effect of CO₂ was eliminated by considering it a confounding variable, and so the Wilcoxon Rank-Sum test for stratified data was used. The effect of DIDS was found to be highly significant, with p < 10⁻⁴. Thus, the presence of DIDS in culture raises pHᵢ significantly. Pairwise t tests (assuming unequal variances, since all F tests showed significant difference in variances) performed at each of the three CO₂ levels showed highly significant differences in mean pHᵢ at each CO₂ (all p < 10⁻⁴). Thus, pHᵢ is raised by DIDS even in 5% CO₂; however, the effect is much greater at lower CO₂, with pHᵢ being very high at 1.7 and 0.8% CO₂ in the presence of DIDS (Fig. 2).

Presence of Functional HCO₃⁻/Cl⁻ Exchange at Each Stage—HCO₃⁻/Cl⁻ exchange was detected by replacing the medium with Cl⁻-free medium and determining if there was an increase in pHᵢ (as described under "Materials and Methods"). Fig. 3 shows the results of these experiments at the one-cell, two-cell, morula, and blastocyst stages. At each stage, switching to Cl⁻-free medium resulted in a marked increase in pHᵢ; pHᵢ then stabilized at a new higher level or peaked and then slowly decreased (as in Fig. 3A). The origin of the decrease is unknown but may be due to passive flux of acid or base equivalents across the membrane (27). The increase was nearly eliminated by the anion exchange inhibitor DIDS (100 uM) (Fig. 3A). The initial rates of increase (Fig. 3B) in the absence of DIDS are all significantly greater than those with DIDS at the same stage, by one-tailed t tests (p = 0.023, 0.032, 0.004, and 0.021, for one-cell, two-cell, morula, and blastocyst, respectively). The mean maximum net pHᵢ increase (Fig. 3C) in the absence of DIDS are also all significantly greater than those with DIDS at the same stage, by one-tailed t tests (p = 0.015, 0.005, 0.008, and 0.009, for one-cell, two-cell, morula, and blastocyst, respectively).

An increase in pHᵢ upon Cl⁻ removal could potentially be mediated by either HCO₃⁻/Cl⁻ exchange or Na⁺/HCO₃⁻/Cl⁻ exchange. To ensure that we were detecting HCO₃⁻/Cl⁻ exchange, Cl⁻ removal was performed in nominally Na⁺-free medium (Fig. 3). In the absence of external Na⁺, pHᵢ still increased upon removal of Cl⁻ at each stage. The mean initial rates of increase (Fig. 3B) were not significantly different from the control recoveries at each stage (p = 0.89, 0.15, 0.64, and 0.42, respectively, by two-tailed t test). The mean extents of the increase (Fig. 3C) were also not significantly different from control at any stage (p = 0.75, 0.57, 0.53, and 0.10). Therefore, there was no evidence of significant Na⁺/HCO₃⁻/Cl⁻ exchange activity at any stage tested, indicating that the increase in pHᵢ upon Cl⁻ removal at each stage demonstrates that there is HCO₃⁻/Cl⁻ exchange activity present at each stage.

To determine whether there was a change in the initial rate of increase of pHᵢ, or in net increase in pHᵢ, over the course of preimplantation development, a t test (two-tailed) was performed to test for a significant difference between the pooled
results for one- and two-cell stages, and the pooled results for morula and blastocyst stages were pooled after determining that there was no significant difference in either parameter within pooled stages; see “Materials and Methods”). Both the initial rate of increase (Fig. 3B) and the net increase (Fig. 3C) decreased significantly from the one- and two-cell stages to the morula and blastocyst stages (p = 0.039 for initial rate and 0.0015 for net increase).

HCO₃⁻/Cl⁻ (Anion) Exchanger mRNAs in Preimplantation Embryos—Embryos at the one-cell, two-cell, morula, and blastocyst stages were examined by RT-PCR for the expression of mRNAs coding for products of the three known HCO₃⁻/Cl⁻ exchanger genes, AE1, AE2, and AE3. Fig. 4 shows the results of these RT-PCR assays. After 30 cycles of PCR, AE2 is detectable at the one-cell and blastocyst stages, more faintly at the morula stage, and barely at the two-cell stage (Fig. 4A). Neither AE1 nor AE3 is detected at any stage after 30 cycles. Using the more sensitive semi-nested PCR protocol, both AE2 and AE3 mRNAs were detectable during preimplantation development. AE2 was again found throughout the preimplantation period, with the greatest amount of product at the one-cell and blastocyst stages, consistent with the results obtained with 30-cycle PCR. AE3 was present from the two-cell stage through the blastocyst stage, but was weakly detected in only a minority of samples of one-cell stage embryos (Fig. 4B). In contrast, AE1 PCR products were barely detectable, and only in a minority of samples, at the one-cell and blastocyst stages, and were undetectable at the two-cell or morula stages (even when another 10 cycles of PCR were added; not shown). PCR products corresponding to AE cDNAs were never found in negative control samples consisting of the last wash drop which had contained the embryos (Fig. 4B).

To ensure that the PCR products visualized were indeed derived from specific AE mRNAs, we used restriction enzymes to cleave the products and show that the fragments generated were of the expected sizes. Fig. 5 shows these results: the PCR products from embryos all gave the expected restriction fragments, and therefore arise from the designated AE mRNAs.
DISCUSSION

Is HCO\textsubscript{3}/Cl\textsuperscript{−} Exchange Present Throughout Preimplantation Embryo Development?—The data presented here indicate that HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity persists throughout preimplantation embryo development in the mouse. We have demonstrated the presence of HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity at each stage by showing a pH\textsubscript{i} increase upon removal of Cl\textsuperscript{−} from the external medium, which causes Cl\textsuperscript{−} efflux from the cell and hence HCO\textsubscript{3}\textsuperscript{−} influx through any functional HCO\textsubscript{3}/Cl\textsuperscript{−} exchanger (Fig. 3). That this pH\textsubscript{i} rise is due to HCO\textsubscript{3}/Cl\textsuperscript{−} exchange is confirmed by the abolition of the increase by DIDS, and its lack of dependence on external Na\textsuperscript{+}, HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity, by this measure, is highest at the one- and two-cell stages and decreases significantly by the morula and blastocyst stage. In addition, work in progress in our laboratory\textsuperscript{2} indicates that recovery from an induced intracellular alkalosis is dependent on external Cl\textsuperscript{−} and on HCO\textsubscript{3} at each stage of preimplantation embryo, further indicating the presence of functional HCO\textsubscript{3}/Cl\textsuperscript{−} exchanger throughout preimplantation development.

Is HCO\textsubscript{3}/Cl\textsuperscript{−} Exchange Activity Necessary for Preimplantation Development?—Under normal culture conditions (i.e., 5% CO\textsubscript{2}, pH 7.35), inhibition of HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity by the anion exchange inhibitor DIDS does not decrease the proportion of two-cell embryos which reach the expanded blastocyst stage by 70 h of culture (Fig. 1), indicating that DIDS (at 100 \(\mu\)M) is not nonspecifically toxic to embryos. DIDS does, however, significantly raise pH\textsubscript{i} under these conditions (Fig. 2). However, when the external pH during culture is raised by lowering [CO\textsubscript{2}], the presence of DIDS has a dramatic effect on embryo development. More than 70% of two-cell embryos develop to blastocysts even in the highest pH used (at [CO\textsubscript{2}] = 0.8 and 0.4\%), but this decreases to 10–20% when DIDS and high pH are combined. Furthermore, when embryos are exposed to even moderately alkaline conditions, functional HCO\textsubscript{3}/Cl\textsuperscript{−} exchange is necessary for development: at pH 7.8 (2% CO\textsubscript{2}), over 90% of two-cell embryos develop to blastocysts, but this drops to less than 40% in the presence of DIDS. Thus, it appears that HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity is necessary for embryo development in the face of even modest increases in external pH. To our knowledge, while it has been clearly shown that HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity participates in pH\textsubscript{i} regulation and volume regulation in many cell types, this is the first demonstration that HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity is required for viability or development in any tissue or cell.

A rise in pH\textsubscript{i} is the most likely cause of the decreased development observed in low CO\textsubscript{2} when HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity is inhibited. Without DIDS, the mean pH\textsubscript{i} is maintained below 7.2 even when the external pH is well above 8.0. This in itself is indicative of activity of the embryo HCO\textsubscript{3}/Cl\textsuperscript{−} exchanger, whose activation threshold is about 7.2 (1). However, in the presence of DIDS, pH\textsubscript{i} is higher at all CO\textsubscript{2} levels tested (Fig. 2). With the exchanger inhibited, mean pH\textsubscript{i} rises to above 7.5, with the pH\textsubscript{i} in individual embryos rising to above 7.8 (the box plots at each point show the population distribution, see figure legend). The greatest pH\textsubscript{i} increases correlate with the most significantly decreased embryo viability, as can be seen from Figs. 1 and 2. Taken together, the data shown in these figures suggest the possibility that the lethal level of pH\textsubscript{i} is about 7.45; the percentages of embryos in each treatment group which fail to develop is approximately equal to the proportion whose pH\textsubscript{i} values are above 7.45 (although a direct correspondence between those embryos with highest pH\textsubscript{i} and those which fail to develop has not been shown).

The results of culture at 5% CO\textsubscript{2} with DIDS indicate that, under normal culture conditions, HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity is not needed for development in vitro. However, it is likely to be necessary for development in vivo. The pH values of oviducal and uterine fluids have not been determined in the mouse, but in other species where this has been measured, the oviducal fluid surrounding embryos has been found to have a high bicarbonate content and high pH, with up to pH 7.7 measured in the rhesus monkey (28), and 7.8–8.2 in the rabbit (29, and references therein). Thus, while artificial culture conditions may allow embryos to grow in the absence of HCO\textsubscript{3}/Cl\textsuperscript{−} exchange activity, it may be continually needed in vivo at least in the portion of preimplantation development that occurs in the oviduct. It is also clear from Fig. 2 that pH\textsubscript{i} is affected even at 5% CO\textsubscript{2}; this may place a stress on the embryos which would compromise their viability in the face of any additional adverse conditions, even if not lethal by itself.

Are AE Family HCO\textsubscript{3}/Cl\textsuperscript{−} Exchangers Present in Preimplantation Embryos?—Our RT-PCR data indicate that AE mRNAs are expressed in preimplantation mouse embryos. AE2 is apparently expressed throughout preimplantation development. It is detectable using mRNA from the equivalent of as few as 2.5 cells after only 30 cycles of PCR and yields a strong signal after semi-nested PCR. AE3 appears to be expressed at least from the two-cell stage onward, as it is detectable with semi-nested PCR (although not after only 30 cycles). In some one-cell samples, a very weak AE3 signal was seen; we believe that this is probably not indicative of functionally important transcription since the signal was extremely weak and was observed in only a minority of the one-cell embryo samples. AE1 mRNA is not expressed at a significant level in the preimplantation embryo, since we detected only a very weak signal in only a minority of samples after seminested PCR (and even then only at the one-cell and blastocyst stages).

RT-PCR is an extremely sensitive technique. In some cases leaky transcription can occur, in which a very few copies of an mRNA are transcribed nonspecifically. These non-physiological transcripts can potentially be detected by RT-PCR if amplification continues for enough cycles. However, the AE2 and AE3 transcripts which we detected are probably expressed at physiologically significant levels. First, we have shown that the

\footnotesize{\textsuperscript{2}Y. Zhao and J. M. Baltz, unpublished results.
seminal molecular study which we used does not detect leaky transcription of AE mRNA in negative control tissues known not to express significant levels of an AE transcript (see "Materials and Methods"). Second, the transcripts are detected from cDNA derived from very few cells. AE2 message was not detected at every embryo stage indiscriminately, as would be expected for leaky transcription.

AE2 message must necessarily be produced from both the maternal and embryonic genomes, since it is present before and after the two-cell stage, where the overall switch from maternal to embryonic gene expression occurs in the mouse. AE3 would thus seem to be a product of the embryonic genome only. It appears that preimplantation stage mouse embryos make mRNA for at least two members of the AE HCO$_3$/Cl$^-$ exchanger family AE2 and AE3. This makes the polypeptides encoded by the AE2 and AE3 genes good candidates for mediating the pH$_i$ regulatory HCO$_3$/Cl$^-$ exchange activity which we have demonstrated in the preimplantation embryo. It is evident that, of these two anion exchangers, AE2 is the most likely to be responsible for HCO$_3$/Cl$^-$ exchange activity at the one-cell stage: there is robust HCO$_3$/Cl$^-$ exchange activity at the one-cell stage (Fig. 3), but little or no AE3 message detectable at this stage, while in contrast there is a strong AE2 RT-PCR signal. However, it still must be shown directly which AE mRNAs are translated into proteins in the plasma membranes of embryos and that these proteins are responsible for the observed HCO$_3$/Cl$^-$ exchange activity and pH$_i$ regulation.

Conclusions—We have shown that preimplantation mouse embryos express the message for at least two HCO$_3$/Cl$^-$ exchangers, AE2 and AE3. One or both of these may mediate the HCO$_3$/Cl$^-$ exchange activity previously shown to regulate pH$_i$ at the two-cell stage and shown here to exist throughout preimplantation development. Exchanger activity is necessary for maintaining embryo pH$_i$, and in conditions where the external environment is even moderately alkaline, as may exist in the oviduct, embryo development depends on functional HCO$_3$/Cl$^-$ exchange.

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