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# Erythroid carbonic anhydrase and *hsp70* expression in chick embryonic development: role of cAMP and hypoxia

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**Dragon, Stefanie, and Rosemarie Baumann.** Erythroid carbonic anhydrase and *hsp70* expression in chick embryonic development: role of cAMP and hypoxia. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R870–R878, 2001.—In the second half of avian embryonic development cAMP affects major aspects of red blood cell (RBC) function. At *day 13/14,* progressive developmental hypoxia causes the release of norepinephrine and erythroid β-adrenergic receptor stimulation initiates the coordinate induction of adaptive key events of erythroid differentiation like carbonic anhydrase (CAII) and 2,3-biphosphoglycerate synthesis. Although cAMP-dependent regulation of CAII protein synthesis has been described in detail, no data exist about the transcriptional regulation in embryonic RBC. Here we report that after *day 12* of embryonic development, the *caII* mRNA is accumulating. Hypoxic incubation at *day 10* as well as in vitro incubation of isolated RBC with cAMP-elevating agonists strongly induces erythroid *caII* expression. The induction of *caII* occurs fast and does not require new protein synthesis. By screening several late erythroid genes, we could identify *hsp70* as another cAMP-induced gene in definitive RBC. Because *caII* (but not *hsp70*) is also induced by cAMP in primitive RBC, the signal may regulate key events of late primitive and definitive erythropoiesis.

red blood cell; polymerase chain reaction; late erythroid genes; β-globin

DESPITE ITS FUNDAMENTAL ROLE in red blood cell (RBC) physiology, little attention has been paid to the mechanisms that control carbonic anhydrase (CAII) expression in this cell type during embryonic and fetal development. Avian RBC synthesize only one CA isozyme (CAII), which in mature RBC is the second most abundant protein.

During the first 2 wk of embryonic development, CAII activity is low but increases sharply at *day 14/15* of incubation (3). The increase of CAII activity is timely coordinated to changes of blood $P_{CO_2}$. In chick embryos the blood $P_{CO_2}$ level is initially low (<10 mmHg) and rises to about 40 mmHg in late development (32) because gas transfer across the egg shell and membranes is limited by diffusion, which causes both progressive hypercapnia and hypoxia (32). The increase of CAII activity curbs to some extent the negative effects of the high $P_{CO_2}$ on acid-base status of embryonic blood, which is important because respiratory acidosis can impair oxygen uptake by Hb in late development, with ensuing negative consequences for the oxygen supply under conditions of hypoxia.

Interestingly, hypoxia, which develops at the same time as hypercapnia, is the physiological stimulus for upregulation of RBC CAII synthesis in late development (3). Hypoxia causes release of adenosine as well as norepinephrine (NE), which by binding to β-adrenergic and adenosine $A_2$ receptors stimulate cAMP production in embryonic RBC (10, 15). In vitro, the cAMP signal induces coordinate changes of CAII synthesis and other RBC properties that affect oxygen transport by Hb (viz. organic phosphate pattern), and the effects of cAMP are identical to the changes in vivo induced by hypoxia (9–11, 15, 22).

The $P_{O_2}$-dependent regulation of $O_2$ and $CO_2$ transport properties allows adaptation of the red cell function to the conditions specific for each individual embryo (18). This is of advantage because huge interindividual differences exist in the diffusive properties of the egg shell and membranes, which directly affect the time course for developmental blood $P_{CO_2}$ and $P_{O_2}$ changes (26, 35, 36).

Although the enzyme activities of RBC CAII during embryonic development are documented, data about the mRNA expression levels in embryonic RBC are lacking. In the present study, we have assessed the relative changes of *caII* mRNA levels during ontogeny between *day 4* and *day 19*. During this time, there is a complete switch in the composition of circulating erythroid cells from primitive polychromatic RBC (*day 4*) to mature definitive RBC [*day 17* to *day 19*, (4, 29)]. Immature definitive RBC normally enter the circulation around *day 6* as postmitotic cells, which retain their transcriptional activity until *day 16/17* (20, 33). Therefore, the definitive RBC of midterm chick embryos are an excellent model to study signaling events that affect transcriptional activity in the penultimate stages of erythroid development. We tested under in vitro conditions the effect of receptor-mediated stimulation of cAMP production on the *caII* mRNA level and we determined the gene expression of *caII* in embryos subjected to experimental hypoxia. We could show that

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during embryonic development the increased CAII protein synthesis and activity are preceded by induction of *caII* mRNA. In vitro stimulation with cAMP-elevating agonists leads to a rapid and stable increase of *caII* mRNA levels in definitive as well as in primitive RBC.

By screening several erythroid genes we identified the heat-shock protein 70 (HSP70) as an additional gene that is induced by cAMP in definitive RBC. HSP70, which is an abundant protein in mature and immature RBC (1, 23) already has been described to be cAMP-responsive in other cellular systems (5, 27, 28, 31). Because it is known for its ability to catalyze folding and unfolding reactions of proteins during protein synthesis and protein degradation processes, our findings suggest that cAMP modulates novel aspects of gene expression in late erythroid differentiation.

## MATERIALS AND METHODS

Fertilized eggs of White Leghorn chickens were incubated at 37.5°C and 60% relative humidity in a commercial forced-draft incubator for up to 19 days of development. For acute hypoxia, the eggs were transferred after incubation for 10 days in air to 13.6% $O_2$ for an additional 20 h (incubator B5060 EK2; Heräus, Nürnberg, Germany).

To obtain blood, a large extraembryonic vessel was cut and the effluent was aspirated and transferred to cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, pH 7.4, at RT; after *day 12* addition of 2 EU/ml heparin). The RBC were washed three times with cold PBS before use.

*In vitro incubations.* RBC of 11-day-old chick embryos were incubated for up to 16 h at 37°C in a gyratory water bath [cytokrit 4%, Ham's medium F10 (Seromed, Biochrom, Berlin, Germany), supplemented with 20 mmol/l HEPES, 10% fetal calf serum (FCS; Boehringer, Mannheim, Germany), pH 7.4, at 37°C] and varying agonists.

*RNA isolation and analysis.* RNA was isolated using the single-step method by acid guanidinium thiocyanate-phenol-chloroform extraction (6). All extractions were done at least in duplicate, and the extracted RNA amount varied less than 10%. We confirmed by control experiments that in the range of 5 and 150 μg RNA, the recovery was 75–80% (data not shown). The RNA integrity and contamination with DNA was examined by agarose gel electrophoresis. In general, the preparations were without any visible DNA contamination. Between *day 10* and *day 15*, the period when the cell population consist of immature definitive RBC, we extracted constant amounts of 10–14 μg RNA/mg Hb.

*Reverse transcription and PCR.* All RNA samples to be compared on a given gel were reverse transcribed into cDNA at the same time. Denatured total RNA (5 μg) was used as template in a 20-μl cDNA synthesis reaction. The RNA samples were incubated with 100 pmol random $d(N)_6$ primers (Pharmacia, Freiburg, Germany) for 10 min at 60°C, chilled on ice, and incubated for 15 min at reverse transcription. Using a master-mix, per sample was added 1× RT buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ 20 mM dithiothreitol (DTT), 0.5 mM each dNTP] and 200 U Superscript II RNase H-reverse transcriptase (GIBCO-BRL Life Technologies, Karlsruhe, Germany) followed by incubation at 42°C for 60 min and 70°C for 15 min. Aliquots (usually 10–50 ng RNA) of the cDNA reactions were analyzed for gene expression with the appropriate primers in 50-μl PCR reactions (see Table 1). To reduce sample variability, all cDNAs to be analyzed on a given gel were amplified at the same time using a master-mix containing (per sample) 1× reaction buffer (PAN systems, Nürnberg), 1.5 mM $MgCl_2$, 200 μM each dNTPs, 10 pmol of each primer (MWG Biotech, Ebersberg, Germany; see Table 1), and 1.25 U PanScript DNA polymerase (PAN systems, Nürnberg). PCR conditions were 94°C, 2.5 min followed by 18–26 cycles (depending on primers and expression level) of 50–60°C, 1 min; 72°C, 1 min; and 94°C, 1 min. To ensure that the conditions used were within the linear range of PCR amplification, aliquots of the reaction were removed at four increasing cycles and analyzed (see below). As an indicator that the PCR products were taken in the linear amplification range of the PCR, a control reaction was performed using five times the initial template amount at *time* 0 ($t_0$). The samples were analyzed on a 2% agarose gel stained with 0.5 μg/ml ethidium bromide (EtBr). The EtBr fluorescence at 590 nm was analyzed with a video documentation system supplied with integrated software (Bioprint version 6.22; Vilbert Lourmat, Marne la Vallee, France). The fluorescence was quanti-

Table 1. *PCR oligonucleotides*

| Gene/Primer | DNA Sequence (5′ to 3′) | Reference | Corresponds to Nucleotide |
|---|---|---|---|
| *s17*(s) | ACAC CCGT CTGG GCAA CGAC | 21,34 | 62–81 |
| *s17*(as) | CCCG CTGG ATGC GCTT CATC | 21,34 | 190–171 |
| *caII*(s) | GGCG TGAA GTAC GATG CAGA G | 38 | 370–390 |
| *caII*(as) | GGCA GCAG TCCA GTAG GGTC | 38 | 593–574 |
| *hsp70*(s) | CCAT CGGC ATCG ATCT GGGC | 24 | 133–152 |
| *hsp70*(as) | AATA GCAG CTGC TGTG GGCT C | 24 | 659–639 |
| *hsf3*(s) | TGCC TGGC TTCC TGGC CAAG | 25 | 57–76 |
| *hsf3*(as) | TTCT CCAA AGCA ACCA CCTT CC | 25 | 270–249 |
| *ho-1*(s) | GACC GGGA GGAG AAGC ATGG | 13 | −16 to +4 |
| *ho-1*(as) | CGAT CTCT TCCT CCAG AGCA G | 13 | 185–205 |
| *β-globin*(s) | CAGA GGTT CTTT GCGT CC | 7 | 288–306 |
| *β-globin*(as) | TAGG TGCT CCGT GATC TT | 7 | 1443–1425 |
| *gata-3*(s) | CCAC CTCC TCCG CTCA TCAC | 21,37 | 818–837 |
| *gata-3*(as) | CCCG GTGC CGTC TCTT CTCC | 21,37 | 1029–1010 |
| *tfr*(s) | GCGT CGAG CCGA TGTC GTAC | 14 | 116–135 |
| *tfr*(as) | GCCA GCTC GATT CGTC CACG | 14 | 371–352 |
| *h5*(s) | GAGC CTGG TCCT ATCC CCAG | 30 | 3733–3752 |
| *h5*(as) | GGAG ACGT CGGA TGGA GAGC | 30 | 3953–3934 |

*hsf3,* Heat shock factor 3; *ho-1,* heme oxygenase-1; *gata-3,* transcription factor; *tfr,* transferrin receptor; *h5,* histone H5.

fied with the analysis software (Optimas 5.10; Optimas, Seattle, WA).

*Northern blots.* Per sample, 5 or 10 μg of total RNA were separated by electrophoresis through a 1% agarose formaldehyde gel. After staining with EtBr, the RNA was transferred by capillary blotting with 10× saline-sodium citrate (SSC) for 16–20 h onto a neutral nylon membrane (porablot NY amp, Macherey-Nagel, Düren, Germany). The transferred RNA was ultraviolet cross-linked and fixed by baking for at least 30 min at 80°C. The nonradioactive hybridization and luminescence detection procedure (anti Dig-antibody, substrate CDP-Star from Roche Molecular Biochemicals, Mannheim) follows the manufacturer's instructions with some modifications (12). For reprobing, the blots were washed for 5 min in distilled water at room temperature. After equilibrating the membranes in 5× SSC for 20 min, the probe was removed by a 2-min stripping in 0.1% SDS at 95°C.

*Probe labeling.* Digoxigenin-11-dUTP from Roche Molecular Biochemicals was used for labeling by PCR. The reactions were carried out according to the instruction provided by the manufacturer. For labeling cDNA of embryonic RBC, the primers of Table 1 were used.

*Protein synthesis.* To test the effect of NE and heat shock on RBC protein synthesis, cells of 11-day-old chick embryos were incubated with [$^{35}$S]methionine (>1,000 Ci/mmol; ICN, Eschwege, Germany). Usually, 50 μl packed RBC were incubated at 37°C in 400 μl F10 medium with 20 mM HEPES in the presence of 10% FCS at pH 7.4, 1 μM NE ± 10 μM propranolol. After 2 h, the medium was changed to methionine-free MEM(−) medium (GIBCO-BRL Life Technologies) with 20 mM HEPES, pH 7.4. After an additional 2 h, 25 μCi [$^{35}$S]methionine was added to each sample and the incubation proceeded for 2 h. The RBC were lysed in lysis buffer (5 mM Tris, 5 mM $NaN_3$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) and centrifuged for 20 min at 13,000 *g* and 4°C. Aliquots containing 100 μg Hb (determined by the cyanmethemoglobin method) of the supernatants were analyzed by SDS polyacrylamide gel electrophoresis according to Laemmli (19). The gels were pretreated with Amplify (Amersham) and were exposed to X-ray film (Kodak Bio-Max MR, Amersham Life Science, Braunschweig, Germany) for 2–5 days at −80°C. For quantitative evaluation of the rate of nonglobin protein synthesis (>20 kDa), the gels of five different experiments were scanned for 3 h with an electronic autoradiography system (InstantImager, Packard Instrument, Meriden, CT).

*Chemicals.* Analytical grade reagents were purchased from Sigma Chemicals (Deisenhofen, Germany). NE, propranolol, and 5′-(*N*-cyclopropyl)-carboxamidoadenosine (CPCA) were obtained from RBI Biotrend (Köln, Germany).

## RESULTS

*Developmental gene expression of caII, β-globin, and s17.* To determine the relative level of a mRNA species in embryonic RBC we used RT-PCR with equal amounts of RNA (see MATERIALS AND METHODS). The proper proceeding of the PCR reactions was monitored by examining the PCR-amplified products after four increasing cycle numbers by gel electrophoresis. Within the linear range of amplification the products were quantified. As expected from the developmental profile of the CAII enzyme activity (Ref. 3, Fig. 1*B*), we found a rise of the *caII* expression level in the last third of development (Fig. 1*A*). The first significant increase of the *caII* mRNA levels occurs at *day 13* when the

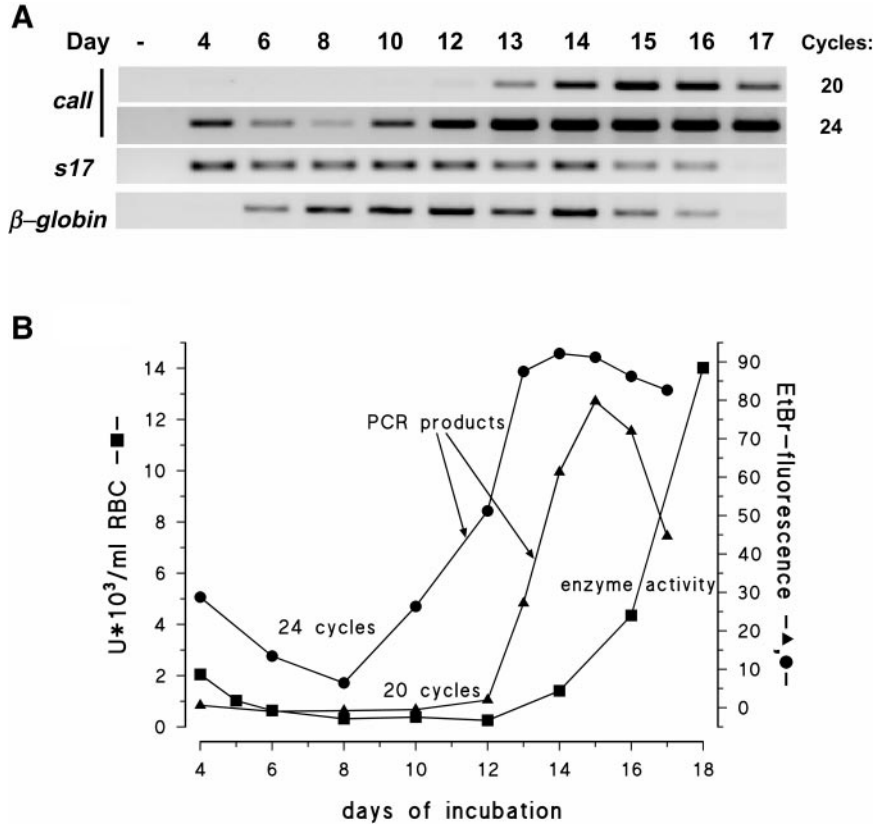


Fig. 1. Gene expression in embryonic red blood cell (RBC) during chick development. For each day the equal amount of reverse transcribed RNA (in general 50 ng; *β-globin* 10 ng) was used for PCR amplification. The experimental procedures of RNA preparation, RT and PCRs were done in duplicate, the ethidium bromide (EtBr)-stained PCR-products of one experiment are shown in *A*. B: quantified EtBr fluorescence of *caII* (means of 2 experiments) and the corresponding enzyme activities of carbonic anhydrase [(CAII), means of 3–10 determinations; (Ref. 2)] during embryonic development.

plasma NE concentration starts to rise (10). At *day 15* the message of *caII* peaks to about 1,000-fold the amount at *day 10* (estimation by PCR dilution series, data not shown). After *day 15*/*16*, the mRNA levels decrease due to the transcriptional shut-down late in development (4, 20) while the CAII enzyme activity is still increasing. The slightly higher mRNA level of *caII* within the primitive RBC population (*days 4* and *6*) corresponds to the higher CAII enzyme activity found at these days (Ref. 3, Fig. 1*B*). In addition, we determined the relative expression of *β-globin* and the small ribosomal protein S17 during development. The *s17* expression shows no gross changes during the switch from primitive to definitive RBC and within the definitive population until *day 15*. The *β-globin* expression is a sensitive measure of the appearance of the first definitive RBC in the embryonic circulation at *day 5–6*. The expression already declines at *day 15* when the *caII* expression is maximal. In conclusion, late in erythroid differentiation when the cellular RNA and dominant mRNA species like *β-globin* are already diminished, there is a selective induction and/or stabilization of the *caII* message in definitive embryonic RBC.

*cAMP-dependent gene expression in embryonic RBC of day 11.* To study gene expression in vitro in response to cAMP induction, we used immature definitive RBC of *day 11*. At this stage, the cells have low CAII enzyme activities. The activity can be increased in vivo by experimental hypoxia (3, 22) or in vitro by β-adrenergic or adenosine receptor activation via formation of cAMP (10, 15). To assess the gene expression of *caII*, we used RT-PCR and Northern blot analysis. We determined the magnitude of changes in gene expression from RT-PCR data by comparing the PCR products of the incubated samples with the control at $t_0$ and with a second control PCR, which contained the fivefold amount of the control cDNA at $t_0$. With aliquots of the cDNA of one original RNA sample, we performed several PCRs to screen the mRNA expression of interesting genes. Because the expression of the small ribosomal protein S17 (*s17*) and *β-globin* stays almost constant during the 4-h incubation period (Figs. 2 and 3), we used the expression level of these genes to verify the specificity of the induction of *caII*.

We assessed the expression levels after an incubation of *day 11* RBC with 1 μM NE for up to 4 h, with or

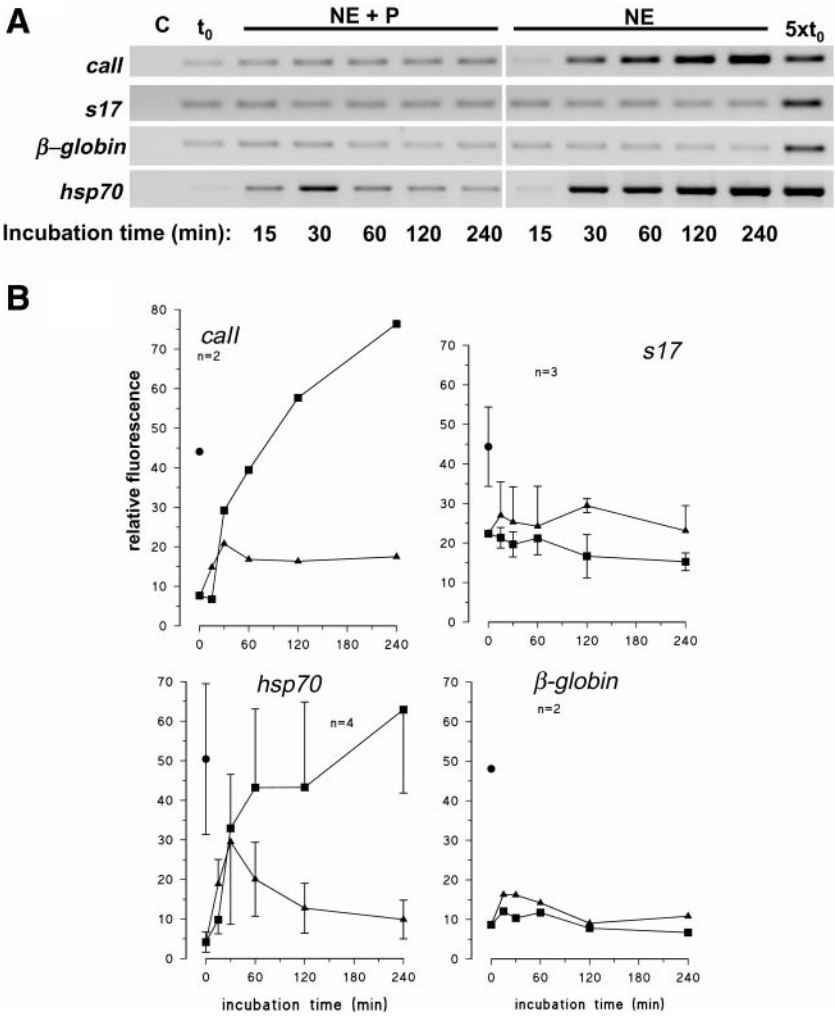


Fig. 2. Expression of *caII*, *s17*, *β-globin*, and *hsp70* in embryonic RBC of *day 11* ($t_0$) and during a 4-h incubation with 1 μM norepinephrine (NE) ± 10 μM propranolol (P). *A*: PCR-products, stained with EtBr. *B*: relative EtBr fluorescences of the PCR products 5× start level ($t_0$; ●), NE + P (▲), and NE (■). Control C, PCR without template; 5× $t_0$, PCR with 5 times the amount of cDNA used for $t_0$ (see also MATERIALS AND METHODS). Presented are the means ± SD of 2–4 independent experiments.

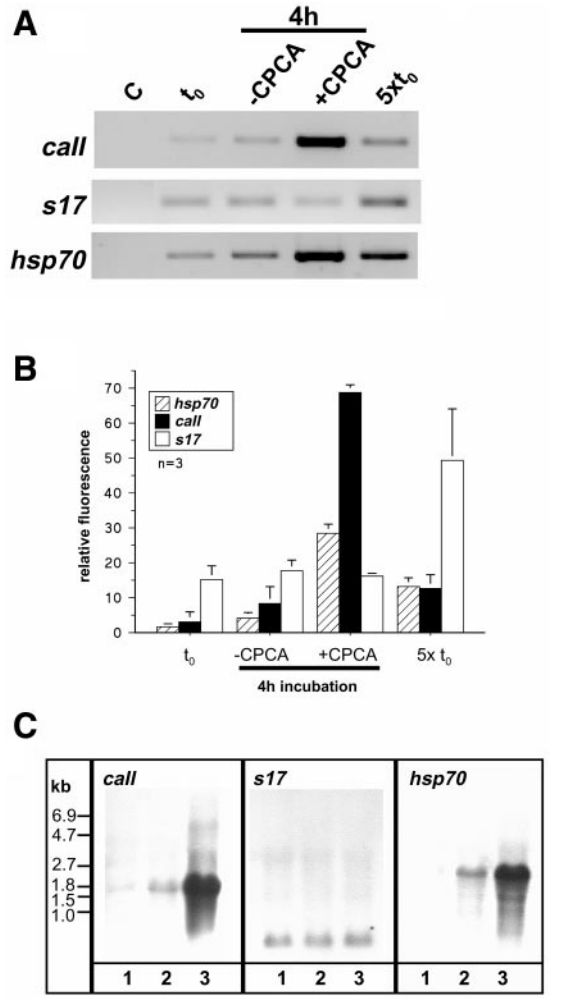


Fig. 3. Expression of *caII*, *s17*, and *hsp70* in embryonic RBC of *day 11* before ($t_0$) and after 4 h of cAMP stimulation with and without 10 μM 5′-(*N*-cyclopropyl)-carboxamidoadenosine (CPCA). *A*: PCR products stained with EtBr. See Fig. 2 for additional label description. *B*: relative EtBr-fluorescences of *caII*, *s17*, and *hsp70*. Presented are the means ± SD of 3 independent experiments. *C*: Northern blot analysis. The blot was loaded with 5 μg RNA per lane (1, $t_0$; 2, −CPCA; 3, +CPCA) and hybridized with probes specific for *s17*, *caII*, and *hsp70* ($n = 2$).

without the specific β-blocker propranolol. In the presence of 1 μM NE, *caII* mRNA levels increased significantly after 30 min of incubation (Fig. 2), whereas changes of CAII activity were not observed before 2 h of incubation (22). The *caII* mRNA increased continuously during the 4-h incubation period, and the induction was suppressed by the β-blocker propranolol.

Because even millimolar concentrations of adenosine are degraded by embryonic RBC within minutes (8), we used the stable agonist CPCA for adenosine $A_2$ receptor activation (Fig. 3). After 4 h of incubation, the induction of the *caII* mRNA was about as effective as in the presence of NE. Northern blot analysis confirmed the results obtained with RT-PCR (Fig. 3*C*).

Because the induction of *caII* transcription is rather fast, we tested whether de novo protein synthesis is needed before transcriptional induction. Inhibition of

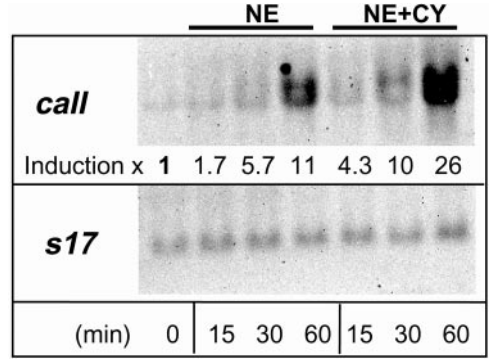


Fig. 4. Induction of *caII* is independent of new protein synthesis. *Day 11* embryonic RBC were incubated for up to 1 h in the presence of 10 μM NE and 50 μM cycloheximide (CY). Northern blot analysis of 10 μg RNA/lane hybridized with probes specific for *s17* and *caII* ($n = 1$). The result was confirmed with PCR ($n = 2$, data not shown).

protein synthesis with 50 μM cycloheximide did not decrease *caII* mRNA expression during 1-h stimulation with NE (Fig. 4). On the contrary, the induction of *caII* mRNA (about 20-fold after 1 h) seems more prominent in the presence of cycloheximide, indicating possibly a slight superinduction of *caII*. In conclusion, efficient induction of *caII* transcription by cAMP presumably requires protein phosphorylation rather than de novo protein synthesis.

*Effect of in vivo hypoxia on caII expression.* After *day 6* of development, a premature increase of the erythroid CAII activity and protein synthesis is observed after acute hypoxic incubation of the embryos (3, 22). To test the effect of acute hypoxia on *caII* mRNA, 10-day-old chick embryos were exposed to 13.6% $O_2$ for 20 h. The gene expression was determined in RBC of individual embryos by RT-PCR. As expected, we observe a prominent increase in the steady-state *caII* mRNA level of RBC from hypoxic embryos compared with the low level of the normoxic control group (Fig. 5, Table 2). The differences in the magnitude of the response to hypoxia presumably reflect the interindividual differences of diffusive properties of the egg shell and membranes (26, 35, 36).

*hsp70 is a cAMP-regulated erythroid gene.* As shown in this and in previous works (10, 15), stimulation of embryonic RBC with cAMP stimulates the synthesis of several proteins aside from CAII (Fig. 6). In addition, we observe an overall increase of the rate of nonglobin synthesis by about 45%.

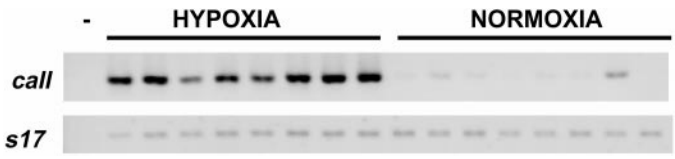


Fig. 5. Hypoxia induces *caII* expression in *day 11* embryonic RBC in vivo. Eggs were either incubated in air for 11 days (normoxia) or they were transferred after 10 days to 13.6% $O_2$ for additional 20 h (hypoxia). The RNA was isolated from RBC of single chick embryos and RT-PCRs were performed with the erythroid RNA of 8 "hypoxic" and 8 "normoxic" embryos (see Table 2). Shown are the PCR products of *caII* and *s17*.

Table 2. *Gene expression of day 11 definitive RBC after hypoxic incubation*

| | Hypoxia ($n$ = 8) | Normoxia ($n$ = 8) |
|---|---|---|
| *caII* | 66.2 ± 23.7 | 1.5 ± 3.6 |
| *s17* | 6.4 ± 2.4 | 7.6 ± 2.1 |

Mean relative ethidium bromide fluorescence of the PCR products ± SD of the products was calculated.

In a first attempt to identify some of the induced proteins, we screened the RNA of cAMP-stimulated *day 11* RBC for the expression of some known erythroid genes. Whereas the gene expression of S17, β-globin, transferrin receptor, GATA-3, histone H5, heat-shock factor-3, and heme oxygenase-1 was unresponsive to cAMP ($n \geq 2$, data not shown), we identified the HSP70, one of the major nonglobin proteins in chicken reticulocytes (23), as a cAMP-responsive gene in definitive embryonic RBC. During a 4-h in vitro incubation of *day 11* RBC, the *hsp70* expression was increased significantly by either β-adrenergic or adenosine receptor activation with a similar time course of expression as observed for *caII* (Figs. 2 and 3). The transcript size of *hsp70* (about 2.6 kb; Fig. 3*C*) agrees with published data (24). We also tested the heat-shock response of gene expression for *s17*, *caII*, and *hsp70* (Fig. 7). As expected, only *hsp70* mRNA showed a marked increase in expression after 30 and 60 min of heat shock at 45°C, whereas mRNA levels of *caII* and *s17* remained unaffected. This suggests different modes of transcriptional control of *hsp70* during heat shock and cAMP stimulation.

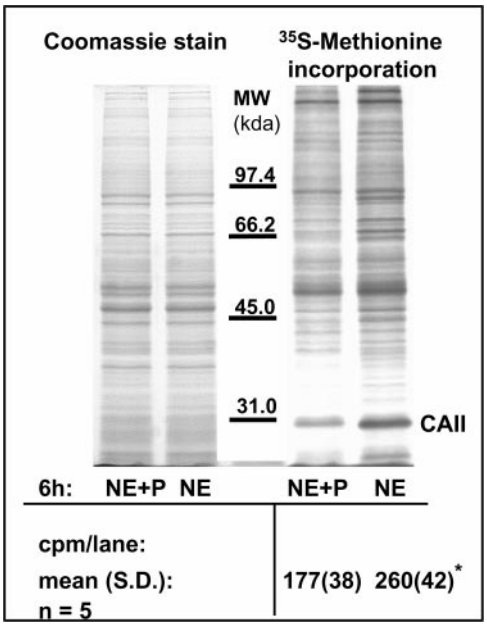


Fig. 6. Protein synthesis after 6 h incubation with 1 μM NE and 10 μM propranolol (NE + P) in RBC of 11-day-old chick embryos. The cytosolic proteins [100 μg hemoglobin (Hb)/lane] were separated by SDS-PAGE and the radioactive labeling with [$^{35}$S]methionine of the proteins synthesized in the last 2 h of incubation was analyzed. *The radioactive incorporation (in cpm/lane) of the NE-treated sample was significantly higher than the sample treated with NE and propranolol (Student's $t$-test, $P < 0.001$ for $n = 5$).

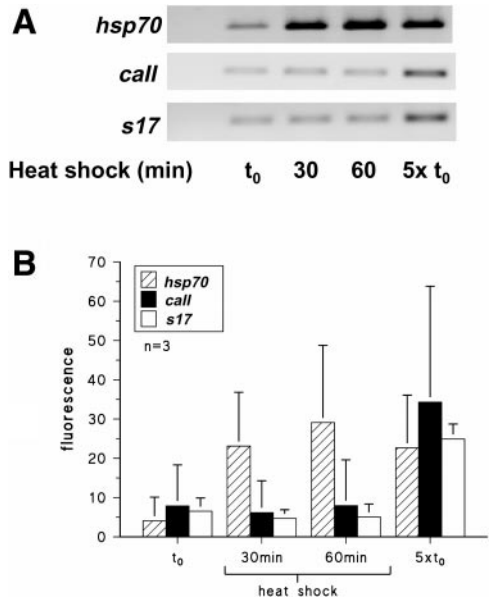


Fig. 7. Expression of *caII*, *s17*, and *hsp70* mRNA in embryonic RBC of *day 11* before ($t_0$) and after heat shock (45°C) for 30 and 60 min. *A*: PCR products stained with EtBr. *B*: relative EtBr-fluorescence, $n$ = 3, means ± SD. See Fig. 2 for additional label description.

*cAMP-dependent caII expression in primitive RBC.* Because primitive RBC of younger embryos possess essentially the same functional cAMP signal transduction system as definitive RBC (2), we wanted to know whether primitive RBC from *day 5* embryos regulate the transcription level of *caII* and *hsp70* by cAMP. During in vitro incubation with 1 μM NE, the primitive RBC upregulate their steady-state level of *caII* mRNA to about the same extent as definitive RBC (Fig. 8). The lack of response to hypoxia at this day in vivo may be tied to insufficient formation of adenosine-NE. In contrast, the *hsp70* expression level, which showed a substantial variability between the four cell pools analyzed, is not significantly altered by cAMP. To assess the presence of definitive RBC in the RBC sample from *day 5* embryos, we checked expression of *β-globin*, which is specific for the definitive lineage. As expected, we observed almost no *β-globin* expression in *day 5* RBC compared with the high erythroid expression level at *day 11*.

## DISCUSSION

The study presents data that give evidence for the transcriptional activation of late erythroid genes, notably CAII, in response to a cAMP signal within a physiological context, the developing chick embryo. We could show that *1*) the developmental profile of erythroid *caII* expression parallels the course of CAII enzyme activities and the rise of plasma NE concentration after *day 13*/*14* of embryonic chick development, *2*) the expression of *caII* is significantly upregulated in vivo by experimental hypoxia at *day 10*/*11*, a process that causes an earlier release of NE into the

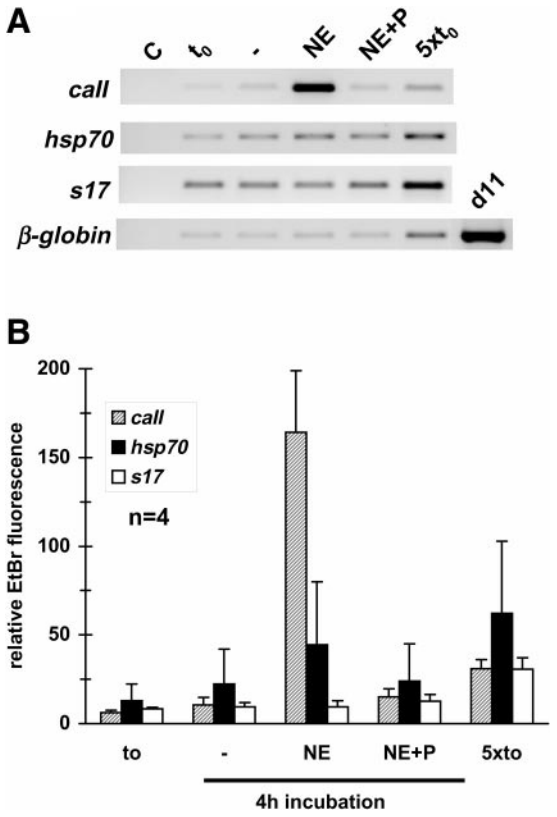


Fig. 8. Expression of *caII*, *s17*, *β-globin*, and *hsp70* in primitive embryonic RBC of *day 5* before ($t_0$) and after 4 h of incubation without agonist (−), with 1 μM NE (NE) or with 1 μM NE and 10 μM propranolol (NE + P). Shown are the EtBr-stained RT-PCR products of one experiment (*A*) and the relative EtBr-fluorescence of the PCR products (*B*), $n = 4$, means ± SD. d11, RT-PCR product of erythroid RNA derived from a *day 11* chick embryo. See Fig. 2 for additional label description.

circulation, *3*) a fast transcriptional activation of *caII* is obtained by in vitro incubation of embryonic definitive and primitive RBC with cAMP-elevating agonists, and *4*) *hsp70* is a further cAMP-regulated gene in definitive but not in primitive RBC of the chick embryo.

Throughout two-thirds of avian embryonic development, the circulating erythroid cells are not fully differentiated and accomplish very late maturation steps like mitochondrial and ribosomal degradation and complete nuclear shut-down in the last days before hatching (16, 20). Furthermore, the immature RBC are able to respond to hormones produced by the embryo during hypoxia and they adapt their transcription pattern and RBC function to changing respiratory parameters (e.g., $Po_2$). Therefore, the circulating erythroid cells of the chick embryo combine constitutive processes of differentiation with adaptive processes on the level of gene expression, which allow a sensible reaction toward physiological changes in an environment that may impair the survival of the avian embryo.

*Erythroid caII expression during embryonic chick development.* In view of the known developmental profile of erythroid CAII activity, the present data of *caII* mRNA levels are an important supplement but not surprising per se. However, in contrast to *β-globin*, which is vital for oxygen transport and which is already fully transcribed in definitive RBC when they appear in the circulation, the *caII* mRNA expression is flexible and easily adjusted to changing physiological conditions. Under normal conditions, the induction of *caII* expression is initiated at *day 13*/*14* and the mRNA level is maximal at *day 15* (Fig. 1), which is close to the developmental stage when the transcriptional shutdown takes place (20). On the other hand, the *caII* induction can be initiated at an earlier developmental stage either in definitive cells [by hypoxic incubation at *day 10*, see Fig. 5, (3, 22)] or in primitive RBC (by in vitro incubation at *day 5*, see Fig. 8). In conclusion, cAMP-dependent *caII* induction shows remarkable flexibility with respect to the differentiation state of erythroid cells.

*cAMP-dependent caII expression in primitive and definitive RBC.* The induction of *caII* expression at *day 11* can readily be initiated in vitro by activating the cAMP signal transduction system. The two characterized functional receptor systems [$\beta_1$-adrenergic and adenosine $A_2$ receptors, (10, 15)] produce within minutes a huge and stable cAMP signal in primitive and definitive RBC (2), which in turn activates *caII* gene expression first visible 15 min after induction (Figs. 2 and 4). The subsequent drastic further rise in expression leads to erythroid CAII activities that are comparable to activities observed in vivo (3, 22), which underlines the validity of the processes of our in vitro model. Because protein synthesis is not required for transcriptional activation, we speculate that phosphorylation of the cAMP-responsive transcription factor cAMP-responsive element-binding protein is needed for promoter activation of *caII*.

In vivo, the first measurable increase in CAII activity during chronic hypoxia is found after *day 6* when definitive RBC are already present in the circulation. In vitro, primitive RBC of *day 5* can readily respond to the cAMP signal with an increase of *caII* mRNA. Thus the early avian embryo is not yet able to stimulate the production of NE or adenosine in response to hypoxia. However, both agonists are present at this developmental stage [NE in the yolk sac and embryo (17); adenosine is universally present]. For this reason, we assume that the hormones may have some role in primitive erythroid differentiation.

*hsp70 as a cAMP-regulated erythroid gene.* By screening the cAMP-dependent expression of several known erythroid genes we identified *hsp70* as a cAMP-induced gene in definitive but not in primitive RBC. Like in other cellular systems, the gene expression is heat-shock sensitive in definitive RBC of *day 11* (Fig. 7). Whereas in definitive RBC the time course of cAMP-dependent expression parallels exactly the induction of *caII* (Fig. 1), primitive RBC failed to upregulate *hsp70* by cAMP (Fig. 8). Apparently, primitive RBC have a different mode or hierarchy of *hsp70* regulation (but not for *caII* gene regulation), which is clearly distinct from definitive RBC.

The function of HSP70 during the differentiation of erythroid cells is obvious, because the reorganization

and destruction of cellular organelles in late differentiation require extensive assistance in folding and unfolding of proteins. In addition, because we observe a significant increase of protein synthesis after cAMP stimulation in embryonic RBC (Fig. 6), HSP70 might also be needed for the folding of de novo synthesized proteins.

*Perspectives*

cAMP regulates in a flexible manner the transcription of two late genes, CAII and HSP70, in definitive RBC of the chick embryo. Whereas CAII is a protein that is specific for proper erythroid function, HSP70 plays a mandatory role for the proper implementation of the specific cell functions. Apparently, the cAMP signal addresses a broad range of target proteins in erythroid cells. Future work should clarify to which extent cAMP-dependent signaling is also involved in other processes characteristic for late erythroid differentiation, viz. mitochondrial and ribosomal breakdown or nuclear condensation. We speculate, that hormonal activation via cAMP could speed up the last steps of RBC differentiation in embryonic development and/or in adult erythropoiesis.

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## REFERENCES


1. **Banerji SS, Laing K, and Morimoto RI.** Erythroid lineage-specific expression and inducibility of the major heat shock protein *hsp70* during avian embryogenesis. *Genes Dev* 1: 946–953, 1987.
2. **Baumann R, Blass C, Götz R, and Dragon S.** Ontogeny of catecholamine and adenosine receptor-mediated cAMP signaling of embryonic red blood cells: role of cGMP-inhibited phosphodiesterase 3 and hemoglobin. Blood 94: 4314–4320, 1999.
3. **Baumann R, Haller E-A, Schöning U, and Weber M.** Hypoxic incubation leads to concerted changes in carbonic anhydrase activity and 2,3-DPG-concentration of chick embryo red cells. *Dev Biol* 116: 548–551, 1986.
4. **Bruns CHO and Ingram VM.** The erythroid cell and hemoglobins of the chick embryo. *Philos Trans R Soc Lond B Biol Sci* 266: 255–305, 1973.
5. **Choi H-S, Li B, Lin Z, Huang E, and Liu AY.** cAMP and cAMP-dependent protein kinase regulate the human heat shock protein 70 promoter activity. *J Biol Chem* 266: 11858–11865, 1991.
6. **Chomczynski P and Sacchi N.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
7. **Dolan M, Dodgson JB, and Engel JD.** Analysis of the adult chicken β-*globin* gene. J Biol Chem 258: 3983–3990, 1983.
8. **Dragon S.** *Hormonelle und metabolische Kontrolle der ATP- und 2,3-BPG-Konzentrationen embryonaler Vogelerythrozyten während der Ontogenese* (PhD dissertation). Universität Regensburg, Germany, 1995.
9. **Dragon S, Carey C, Martin K, and Baumann R.** Effect of high altitude and in vivo adenosine/β-adrenergic receptor blockade on ATP and 2,3BPG concentrations in red blood cells of avian embryos. *J Exp Biol* 202: 2787–2795, 1999.
10. **Dragon S, Glombitza S, Götz R, and Baumann R.** Norepinephrine-mediated hypoxic stimulation of embryonic red cell carbonic anhydrase and 2,3-DPG synthesis. *Am J Physiol Regulatory Integrative Comp Physiol* 271: R982–R989, 1996.
11. **Dragon S, Hille R, Götz R, and Baumann R.** Adenosine 3′,5′-cyclic monophosphate (cAMP)-inducible pyrimidine 5′-nucleotidase and pyrimidine nucleotide metabolism of chick embryonic erythrocytes. *Blood* 91: 3052–3058, 1998.
12. **Engler-Blum G, Meier M, Frank J, and Müller GA.** Reduction of background problems in nonradioactive Northern and Southern blot analyses enables higher sensitivity than $^{32}$P-based hybridizations. *Anal Biochem* 210: 235–244, 1993.
13. **Evans C-O, Healey JF, Greene Y, and Bonkovsky HL.** Cloning, sequencing and expression of cDNA for chicken liver haem oxygenase. *Biochem J* 273: 659–666, 1991.
14. **Gerhardt EM, Chan LN, Jing SQ, Qi MY, and Trowbridge IS.** The cDNA sequence and primary structure of the chicken transferrin receptor. *Gene* 102: 249–254, 1991.
15. **Glombitza S, Dragon S, Berghammer M, Pannermayr M, and Baumann R.** Adenosine causes cAMP-dependent activation of chick embryo red cell carbonic anhydrase and 2,3-DPG synthesis. *Am J Physiol Regulatory Integrative Comp Physiol* 271: R973–R981, 1996.
16. **Grima M and Girard M.** Oxygen consumption by chick blood cells during embryonic and post-hatch growth. *Comp Biochem Physiol A Physiol* 69: 437–442, 1981.
17. **Ignarro LJ and Shideman FE.** Norepinephrine and epinephrine in the embryo and embryonic heart of the chick: uptake and subcellular distribution. *J Pharmacol Exp Ther* 159: 49–58, 1968.
18. **Koller M, Dragon S, and Baumann R.** Control of red cell function of late chick embryos: role of extracellular ATP/AMP and egg size. *Am J Physiol Regulatory Integrative Comp Physiol* 267: R542–R548, 1994.
19. **Laemmli UK.** Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 224: 680–685, 1970.
20. **Landes GM, Villeponteau B, Pribyl TM, and Martinson HG.** Hemoglobin switching in chickens. *J Biol Chem* 257: 11008–11014, 1982.
21. **Leonard MW, Lim K-C, and Engel JD.** Expression of the chicken GATA factor family during early erythroid development and differentiation. *Development* 119: 519–531, 1993.
22. **Million D, Zillner P, and Baumann R.** Oxygen-pressure-dependent control of carbonic anhydrase synthesis in chick embryonic erythrocytes. *Am J Physiol Regulatory Integrative Comp Physiol* 261: R1188–R1196, 1991.
23. **Morimoto R and Fodor E.** Cell-specific expression of heat shock proteins in chicken reticulocytes and lymphocytes. *J Cell Biol* 99: 1316–1323, 1984.
24. **Morimoto RI, Hunt C, Huang S-Y, Berg KL, and Banerji SS.** Organization, nucleotide sequence, and transcription of the chicken hsp70 gene. *J Biol Chem* 261: 12692–12699, 1986.
25. **Nakai A and Morimoto RI.** Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. *Mol Cell Biol* 13: 1983–1997, 1993.
26. **Nakazawa S and Tazawa H.** Blood gases and hematological variables of chick embryos with widely altered shell conductance. *Comp Biochem Physiol A Physiol* 89: 271–277, 1988.
27. **Osaki J, Haneda T, Kashiwagi Y, Oi S, Fukuzawa J, Sakai H, and Kikuchi K.** Pressure-induced expression of heat shock protein 70 mRNA in adult rat heart is coupled both to protein kinase A-dependent and protein kinase C-dependent synthesis. *J Hypertens* 18: 1193–1200, 1998.
28. **Pizurki L and Polla BS.** cAMP modulates stress protein synthesis in human monocytes-macrophages. *J Cell Physiol* 161: 169–177, 1994.
29. **Romanoff AL.** *The Avian Embryo.* New York: MacMillan, 1960.
30. **Ruiz-Carrillo A, Affolter M, and Renaud J.** Genomic organisation of the genes coding for the six main histones of the chicken: complete sequence of the H5 gene. *J Mol Biol* 170: 843–859, 1983.
31. **Takano M, Arai T, Mokuno Y, Nishimura H, and Yoshikai Y.** Dibutyryl cyclic adenosine monophosphate protects mice against tumor necrosis factor-alpha induced hepatocyte apopto-

sis accompanied by increased heat shock protein 70 expression. *Cell Stress Chaperones* 3: 109–117, 1998.
32. **Tazawa H.** Oxygen and $CO_2$ exchange and acid-base regulation in the avian embryo. *Am Zool* 20: 395–404, 1980.
33. **Tobin AJ, Hansen DA, Seftor EA, McCabe JB, and DeCloe J.** Transcription and processing of mRNAs during erythroid development in chickens. In: *Regulation of Hemoglobin Synthesis*, editor by Goldwasser E. New York: Elsevier, 1983, p. 271–280.
34. **Trueb B, Schreier T, Winterhalter KH, and Strehler EE.** Sequence of a cDNA clone encoding chicken ribosomal protein S17. *Nucleic Acids Res* 16: 4723, 1988.
35. **Visschedijk AHJ, Tazawa H, and Piiper J.** Variability of shell conductance and gas exchange of chicken eggs. *Respir Physiol* 59: 339–345, 1985.
36. **Wangensteen OD, Wilson D, and Rahn H.** Diffusion of gases across the shell of the hen's egg. *Respir Physiol* 11: 16–30, 1970/71.
37. **Yamamoto M, Ko LJ, Leonard MW, Beug H, Orkin SH, and Engel, JD.** Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes Dev* 4: 1650–1662, 1990.
38. **Yoshihara CM, Lee JD, and Dodgson JB.** The chicken carbonic anhydrase II gene: evidence for a recent shift in intron position. *Nucleic Acids Res* 15: 753–770, 1987.