

The Mechanism of Osmotic Transfection of Avian Embryonic Erythrocytes: Analysis of a System for Studying Developmental Gene Expression

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Abstract. We have undertaken a study of the mechanism of DNA transfer into primary chicken erythrocytes by a method named osmotic transfection. The cells are subjected to controlled osmotic swelling in NH_4Cl and then ruptured in a lower osmotic strength solution containing DNA and DEAE-dextran. The osmotic rupture results in transient formation of a single hole in the cell membrane, which is followed within hours by recovery of near normal levels of RNA and protein synthesis. The association of DNA with the cells is much greater for ruptured than for unruptured cells or for cells that have been lysed and resealed before DNA is added. Transient formation of pores in the cell membrane is apparently essential for high rates of macromolecular transfer into the cell. DEAE-

dextran increases the amount of DNA associated with the cells, especially after cell rupture. Our understanding of the mechanism has allowed us to extend the application of osmotic transfection to essentially all developmental stages of avian erythroid differentiation. Osmotic transfections were done with plasmids containing the chloramphenicol acetyl transferase (*cat*) gene placed between the chicken β -globin promoter and the 3' β -globin enhancer. The pattern of CAT expression at sequential developmental stages parallels that of the endogenous gene, showing that osmotically transfected cells appear to retain developmental fidelity. The approach provides a convenient, sensitive, and flexible system for the study of transient gene expression as a function of development.

TRANSFECTION of DNA into eukaryotic cells has provided a powerful tool for the study of gene expression in higher organisms, but the mechanisms are not well understood for most approaches. In the course of studying the control of globin gene expression in chicken erythrocytes, we developed a method for transfecting DNA into primary erythrocytes, which was based on controlled osmotic shock of the cells (Hesse et al., 1986). We now explore the details of the transfection mechanism.

Here we examine the relationships between osmotic rupture of cells, binding of DNA, and subsequent expression of genes encoded on that DNA. We show that osmotic rupture of cells in the presence of DNA and DEAE-dextran markedly increases association of DNA with cells, and that this rupture is essential for high levels of gene expression. Cells that have undergone lysis recover transcriptional and translational biosynthetic activity. Our understanding of the mechanism of osmotic transfection allows us to now extend its application to the transfection of primary cells at essentially all stages of avian erythroid development, and we show that transfected cells maintain fidelity of developmental gene expression.

We first developed the osmotic transfection method in order to assess the biological role of regulatory sequences as-

sociated with globin gene expression. In our laboratory and others, chromatin structural features in the neighborhood of these genes have been correlated with transcriptional states (Stalder et al., 1980; McGhee et al., 1981). Analysis of hypersensitive domains within globin chromatin has led to identification of DNA sequences and nuclear factors with demonstrated or presumed regulatory functions (Emerson and Felsenfeld, 1984; Emerson et al., 1985; Jackson and Felsenfeld, 1985). In particular, we have identified a strong tissue-specific enhancer at the 3' end of the adult β -globin gene (Hesse et al., 1986). Our knowledge of the details of the transfection mechanism provides us with a reliable method for the assessment of the function of these elements during development as we show here for the β -globin gene, and provides more general insight into the mechanisms of transfection.

Materials and Methods

Erythrocyte Isolation

Erythrocytes from chicken eggs 3–17 d after laying were collected into 25 ml of PBS (0.726 g of anhydrous Na_2HPO_4 per liter, 0.12 g of anhydrous KH_2PO_4 per liter, 9 g of NaCl per liter) at 22°C. Erythrocytes were

pelleted at 800 g for 5 min at 22°C, washed once in 45 ml of PBS, and resuspended in PBS at $\sim 10^8$ cells per ml. 1-ml aliquots were distributed into 1.5-ml Eppendorf microfuge tubes (Brinkmann Instruments, Inc., Westbury, NY) and used within 3 h.

Swelling Step of Osmotic Transfection

Reagents were sterile and used at 22°C unless otherwise specified. Cell suspensions (10^8 cells/ml) from the erythrocyte isolation procedure were centrifuged in a microfuge 12 (Beckman Instruments Inc., Palo Alto, CA) for 1 min at 12,400 g. Supernatants were aspirated and pellets were softened by brief, gentle mixing in a VWR Vortex (model K-550-G; Scientific Industries, Inc., Bohemia, NY). Each pellet was resuspended in 1 ml of NH_4Cl (pH 7) solution at a concentration specified in individual experiments and incubated at 22°C for various times. In the standard osmotic transfection, the concentration of NH_4Cl was 250 mM. Unless otherwise specified, the length of the swelling step incubation was 106 min for 3- and 4-d embryonic red cells, 67 min for 5-d red cells, 40 min for 9-d and older embryonic red cells. At the end of the swelling step incubation, the cells were pelleted by centrifugation at 10,500 g for 1 min. The NH_4Cl solution was aspirated, and the cell pellet was softened by gentle vortexing. This swelling step was followed immediately by the transfer step described below.

Transfer Step of Osmotic Transfection

Each cell pellet was resuspended in 0.5 ml of DEAE-dextran-DNA solution (McCutchan and Pagano, 1968) (67% [vol/vol] L15 [Liebovitz] medium without phenol red [Flow Laboratories Inc., McLean, VA], 0.05 M Tris-HCl, pH 7.45, 300 μg of DEAE-dextran [Pharmacia P-L Biochemicals, Piscataway, NJ; $\approx 5 \times 10^5$ D] per ml, and 1–3 μg of DNA per ml), which was prepared 5–120 min before use. This cell suspension was incubated 10 min at 37°C, after which the cells were pelleted at 10,500 g for 1 min, washed once in 100% L15 medium, repelleted at 10,500 g for 1 min, resuspended in 0.25 ml of culture medium (92% [vol/vol] L15 medium, 5% FCS [Flow Laboratories Inc.], 2% chicken serum [GIBCO, Grand Island, NY] and 100 μg of kanamycin sulfate [GIBCO] per ml), and incubated, with tubes tightly capped and lying on their sides, at 37°C for 48 h.

Assay for Chloramphenicol Acetyl Transferase

Cells were harvested for assay 48 hr after transfection. Erythrocytes were pelleted at 10,500 g for 1 min, washed once in 1 ml PBS at 22°C, pelleted, resuspended in 0.16 ml of 100 mM NH_4HCO_3 , dispersed by vigorous mixing in a VWR Vortex (model K-550-G; Scientific Industries, Inc.) and lysed by incubation at 37°C for 5 min followed by three cycles of freezing and thawing (dry ice/ethanol bath, 37°C bath). The lysed cells were pelleted at 10,500 g for 5 min. The supernatant was removed and assayed for chloramphenicol acetyl transferase (CAT)¹ activity. To measure CAT activity within the linear range of the assay, a suitable fraction of each cell extract was used in the CAT assay. The assay procedure used has been described (Gorman et al., 1982; Lopata et al., 1984) except that each assay contained 5.6 mM acetyl coenzyme A and 0.25 μCi [¹⁴C]chloramphenicol.

Hemoglobin Assay

The concentration of hemoglobin (Hb) was determined spectrophotometrically by measuring the absorbance at 412 nm. The molar extinction coefficient used for Hb was 1.61×10^5 liter/mole-cm.

Cell Number Determination

Cell number was determined by centrifugation of 1 ml of cell suspension at 10,500 g for 1 min, resuspension of the pellet in 1 ml 100 mM NH_4HCO_3 (pH 8), centrifugation at 10,500 g for 1 min, and spectrophotometric reading of a dilution of the hemoglobin-containing supernatant at 412 nm. Based upon the extinction coefficient of Hb, cell volume, and cellular Hb content for the mixed population of cells present at each developmental stage (Romanoff, 1960), the cell number was calculated from the absorbance. Cell number equals the optical density (412 nm) times 8.52×10^6 for 3-d RBC, 6.45×10^6 for 4-d RBC, 5.34×10^6 for 5-d RBC, 7.70×10^6 for 9-d RBC, 8.52×10^6 for 10-d RBC, and 9.31×10^6 for 11-d and older embryonic erythrocytes.

1. Abbreviation used in this paper: CAT, chloramphenicol acetyl transferase.

Polyacrylamide-Triton X-100-Urea Gels

Peptide chains in lysates of erythrocytes were qualitatively analyzed using an electrophoresis system described previously for separation of globin chains (Alter et al., 1980). In brief, the gel solution consisted of 4 vol 60:0.4% acrylamide/bis-acrylamide, 1 vol of glacial acetic acid, 15 vol of 8 M urea, 0.4 vol Triton X-100, and 0.15 vol of 10% ammonium persulfate. The electrophoresis buffer was 5% acetic acid. Electrophoresis was for 17 h at 8.5 mA, run at constant current.

Results

Correlation of DNA Transfection and Expression with Cell Swelling and Rupture

Primary circulating erythrocytes from 11-d chick embryos express genes in DNA introduced into the cells by the conventional DEAE-dextran method of McCutchan and Pagano (1968). In preliminary experiments, the CAT expression vector, pRSVcat, in which the Rous sarcoma virus long terminal repeat is fused to the *cat* gene, was introduced into the erythrocytes using this method. An extract prepared from the transfected cells 48 h after the DEAE-dextran-DNA treatment contains CAT. The tonicity of the DEAE-dextran-DNA solution (transfer step solution) is correlated with the subsequent expression signal (Fig. 1). When we modified the conventional DEAE-dextran-DNA solution by increasing dilutions of the media, we found that expression 48 h later was increased, peaking at a tonicity of 140 mosM (Fig. 1).

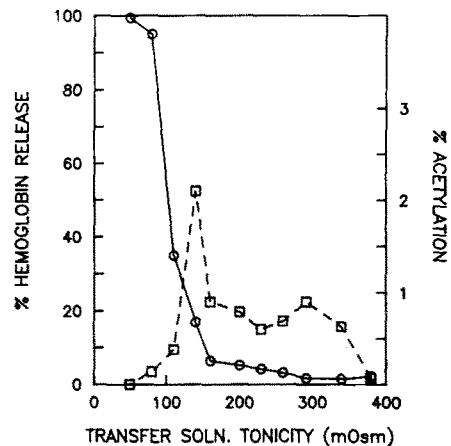


Figure 1. Hb release and transient expression as a function of transfer step solution tonicity. 11-d embryonic erythrocytes (5×10^7 cells) were suspended in 500 μl L-15 medium (without phenol red) at various dilutions, 50 mM Tris-Cl (7.4), 1 $\mu\text{g}/\text{ml}$ RSVcat DNA, and 300 $\mu\text{g}/\text{ml}$ DEAE-dextran. The L-15 medium concentrations were 1, 0.85, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1, and 0 times unit strength (300 mosM). For an additional point (furthest left), the L-15 medium strength was zero and the Tris-Cl concentration was 30 mM (50 mosM) rather than 50 mM (83 mosM). The cells were incubated in these solutions for 10 min \times 37°C and centrifuged at 12,000 g for 1 min at 22°C. The supernatant was removed and assayed for Hb concentration spectrophotometrically. The cell pellet was washed once in L-15 and resuspended in culture media for 48 h at 37°C. At that time, cell lysates were prepared and assayed for CAT. The figure plots the percentage acetylation (\square) in the standard CAT assay (see Materials and Methods) as a function of the total milliosmolar tonicity (media and buffer) in the transfer solution. The percentage Hb release (\circ) in the transfer solution is also plotted for each tonicity.

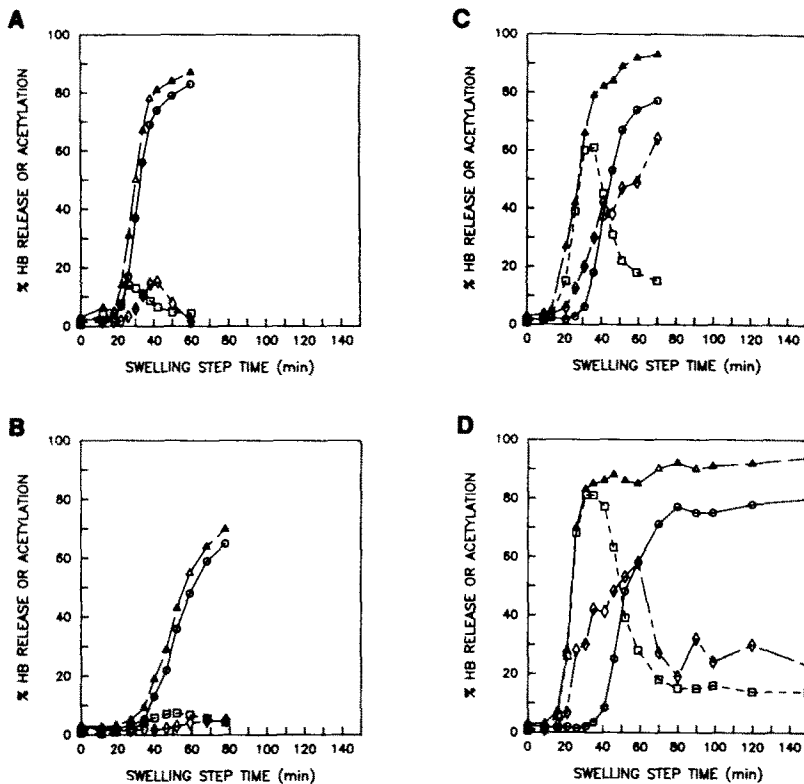


Figure 2. Hb release and transient gene expression as a function of swelling step time at various tonicities of the swelling step solution. Embryonic erythrocytes (1.24×10^8 cells) from 12-d chicken eggs were resuspended in a swelling step solution composed of 150 mM NH_4Cl (A), 100 mM $\text{NH}_4\text{Cl}/50$ mM NaCl (B), 200 mM NH_4Cl (C), or 250 mM NH_4Cl (D), and incubated at 22°C for various swelling step times. At the end of this interval, the cells were pelleted at $10,500$ g for 1 min. The swelling step supernatant was removed and assayed for Hb (\circ). The cell pellet was resuspended in the standard transfer step solution (see Materials and Methods) containing DEAE-dextran and pRSVcat DNA for 10 min at 37°C . The cells were pelleted at $10,500$ g, the supernatant was saved, and assayed for Hb (\square), and the pellets were washed once in L15 medium. Each cell pellet was resuspended in culture media for 48 h, after which cell extracts were assayed for CAT (\diamond). CAT activity is normalized to 10^8 cells with a 1-h incubation in the standard CAT assay (see Materials and Methods). The sum of swelling step hemoglobin release and transfer step hemoglobin release is expressed as total hemoglobin release (Δ) in the osmotic transfection procedure.

We found that we could swell the cells while maintaining a physiologic ionic strength, by using NH_4Cl in a swelling step administered immediately before DEAE-dextran (transfer step) treatment (see Discussion). Cells were swollen by incubation in 150 mM NH_4Cl , pelleted, and resuspended in a DEAE-dextran-DNA solution. Resuspension of the cells in the DEAE-dextran-DNA solution is termed the transfer step, and we use media diluted to 0.66 strength as our standard concentration in this step (see Materials and Methods). Cell rupture and Hb release occurred and were monitored by spectrophotometric assay of the swelling and transfer step supernatants. The extent of Hb release increases with the length of the swelling step incubation (Fig. 2 A). The subsequent expression signal increased dramatically with this method of swelling the cells.

To increase reproducibility of the method, we were interested in broadening and delaying the time interval of the swelling step that yielded peak expression. We found that this could be achieved by incubating cells during the swelling step in hypertonic NH_4Cl solutions (200 or 250 mM) or in mixtures of NH_4Cl and NaCl (Fig. 2, B–D). After various times of swelling, cells were transferred to the DEAE-dextran-DNA solution. Subsequent CAT expression and Hb release in the swelling and transfer steps showed the following features. (a) The expression peak is broader and higher with swelling in hypertonic NH_4Cl solutions. (b) The expression peaks shift with the Hb release curves. (c) Optimal expression for each time course is achieved when total Hb release is on average 80% (Fig. 2, A–D). (d) In those time courses in which the Hb release in the transfer step is large (Fig. 2, C and D), the expression is large. (e) Even at or near 100% Hb release and cell rupture, expression remains above that of the point for zero swelling step time.

The above experiments were carried out with erythrocytes from 9- or 12-d-old embryos, both yielding similar results (see below, Fig. 4).

Cell Membrane in Osmotic Transfection

It is known that osmotic rupture of human erythrocytes does not cause complete membrane disruption, but results in transient formation of a single hole in the plasma membrane of each cell (Baker, 1967; Seeman, 1967; Huhn et al., 1970; Yee and Mel, 1978; Lieber and Steck, 1982a, b). We find that avian erythrocytes, like their nonnucleated human counterparts, osmotically rupture at a single site. Erythrocytes isolated from 9-d-old embryos were subjected to the standard osmotic transfection procedure for 9-d embryonic erythrocytes (see Materials and Methods), but with 1% glutaraldehyde in the transfer step. The glutaraldehyde cross-links the Hb molecules to each other (Baker, 1967; Yee and Mel, 1978). The cells were examined in a phase-contrast microscope (Fig. 3). Cells aligned appropriately within each field ($\sim 25\%$ of the cells) reveal a single plume of cross-linked Hb at the site of efflux. Of 300 cells examined, none had more than one plume.

Osmotic Transfection of Cells of Different Developmental Age and Size

We previously reported studies of osmotic transfer of DNA into 5-, 9-, and 12-d embryonic erythrocytes. We were interested in extending the capability of DNA transfer to a wider range of the erythroid developmental progression. We have found in this and our previous studies that the cells from different developmental stages, which vary in cell volume, require different times of swelling step incubation to achieve

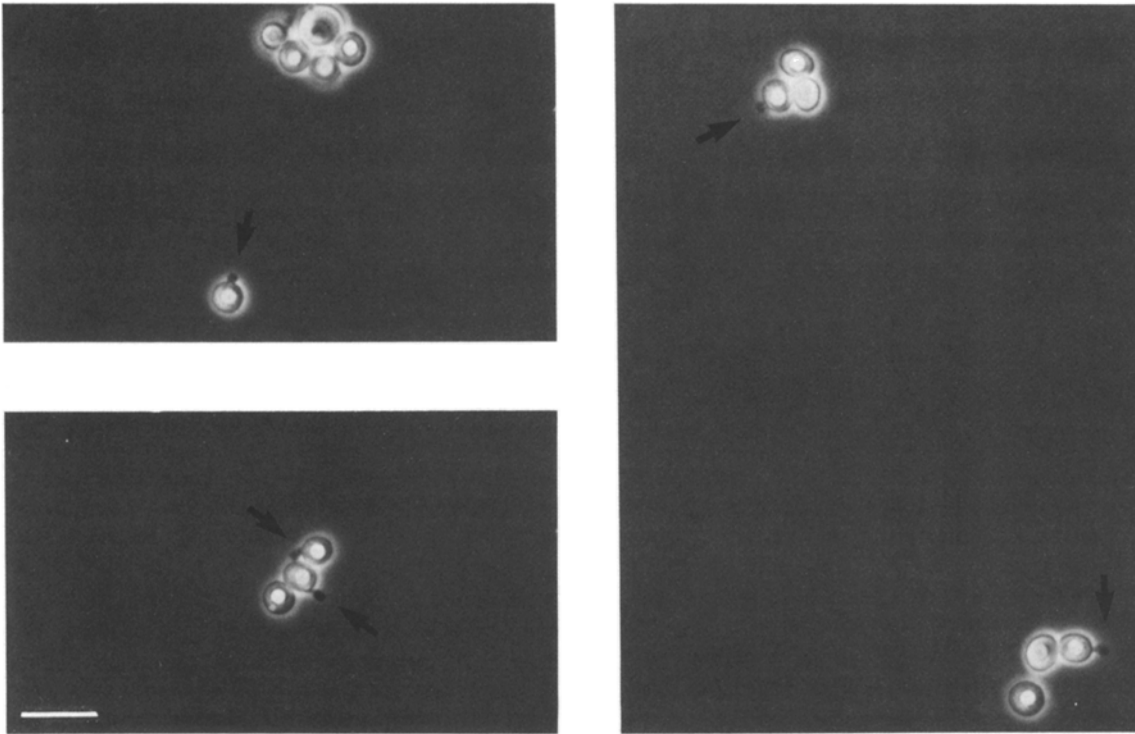


Figure 3. Single site of hemoglobin efflux in osmotically transfected 9-d embryonic erythrocytes. Embryonic erythrocytes were subjected to a standard osmotic transfection for 9-d cells except that 1% glutaraldehyde was present in the transfer step solution and DEAE-dextran and DNA were omitted. Photographs were taken 1 min after resuspension of the cells in the transfer step solution. Bar, 20 μ m.

peak expression. Younger embryonic erythrocytes are larger and require a longer swelling step incubation in order to reach the peak of CAT expression (Fig. 4, A-D, and Fig. 5). The relationship between normal cell volume (V_0) and lytic

cell volume (V_L) relative to t^* , the time for half of the cells to lyse, is given by the equation: $V_0/2 ((V_L/V_0)^2 - 1) = kt^*$, where k is the composite permeability coefficient for NH_4^+ and Cl^- and reflects the mode by which NH_4Cl enters

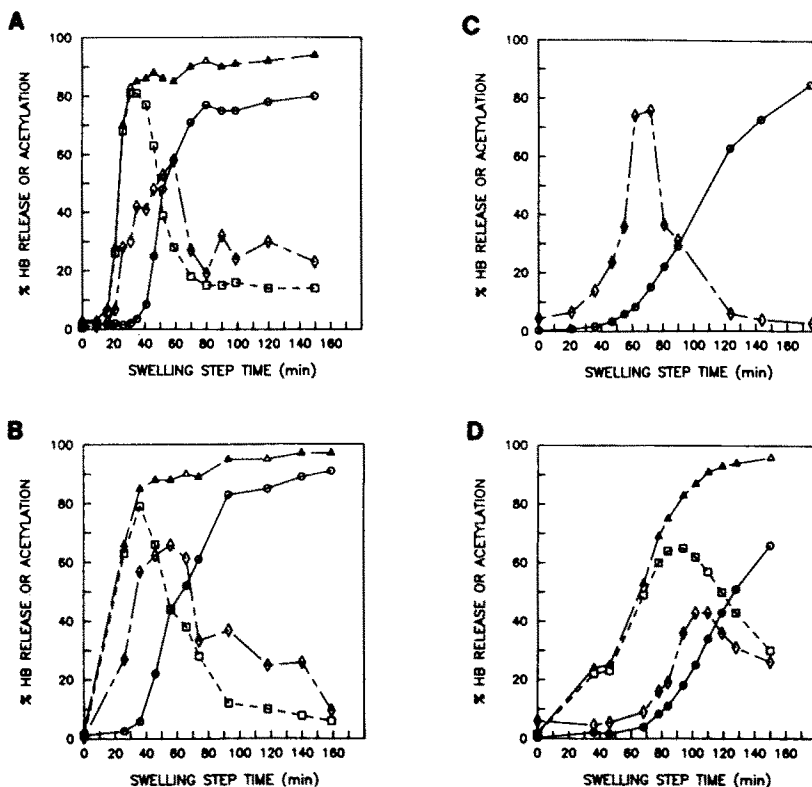


Figure 4. Hb release and gene expression as a function of swelling step time for embryonic erythrocytes at different developmental ages. Chicken embryonic erythrocytes from 12- (1.24×10^8 cells, A), 9- (9.7×10^7 cells, B), 5- (7.2×10^7 cells, C), and 4-d (7.0×10^7 cells, D) were resuspended in 250 mM NH_4Cl at 22°C. At various times, the samples were pelleted at 10,500 g for 1 min and resuspended in the standard transfer step solution for 10 min at 37°C. Hb release in the swelling step (\circ) and transfer step (\square) were measured spectrophotometrically (see Materials and Methods). The sum of Hb release in these two steps is the total Hb release (Δ). The cells were then pelleted, washed once in LI5, and resuspended in media (see Materials and Methods). At 48 h after transfection, the cells were harvested and assayed for CAT (\diamond) as described in Materials and Methods. Chloramphenicol acetylation values shown are normalized to 1×10^8 cells, and the activity values in C are 0.33 times their actual values. Transfer step Hb release was not measured for 5 d cells.

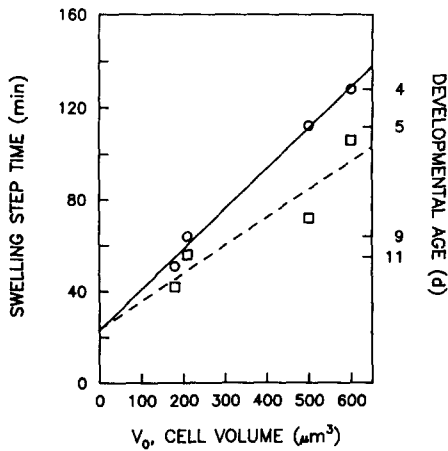


Figure 5. Swelling step time for half-maximal Hb release and maximal gene expression as a function of normal cell volume at various developmental ages. The swelling step time corresponding to 50% Hb release in the swelling step (○, —) and to maximal CAT expression (□, - - -) in Fig. 4 (A, B, C, and D) are plotted as a function of cell volume (Romanoff, 1960). The solid line also relates developmental age and cell volume.

(Jacobs, 1952; Lieber and Steck, 1984). For erythroid cells, which contain the anion exchange protein, Band 3, k is predominantly dependent on the number of Band 3 molecules per unit surface area and is relatively constant from embryonic days 4 to 18 (Chan, 1977). The ratio V_t/V_o is relatively constant along this developmental series and was determined to be ~ 2 from hypotonic hemolysis curves (data not shown); for two ages of cells, $V_o(1)/V_o(2) = t^*(1)/t^*(2)$. Therefore, 5-d cells, which have 2.4 times the starting volume of 9-d cells, require nearly twice as long to swell to maximum volume and achieve half-maximal lysis (Fig. 5). As a result, the average cell size at any developmental stage can be used to predict the approximate swelling step incubation length appropriate to yield subsequent peak expression.

It should be noted that transfection of 4-d-old embryonic erythrocytes is more variable than that of cells at later stages. Some 4-d cells have proven unusually resistant to lysis, while others display high levels of CAT activity when they are directly transfected with DEAE-dextran-DNA without a prior swelling step (see below). In part, this reflects the considerable variation in embryonic erythroid cell populations between 4 and 5 d of development, and variation in the fragility of the cells due to details of cell isolation procedures.

Role of DEAE-Dextran in Osmotic Transfection

To determine if DEAE-dextran is an essential component for DNA transfer, 9-d erythrocytes were osmotically transfected with pRSVcat, but without DEAE-dextran in the transfer step. A low but significant level of CAT expression was observed (not shown). Therefore, DEAE-dextran is not essential, but markedly increases DNA transfer. It seemed likely that the polyanionic DNA and cell membrane might associate via electrostatic interaction with the polycationic DEAE-dextran. To test this, binding of ^3H -pBR322 plasmid DNA to 9-d erythrocytes was measured under various transfection conditions (Table I). Labeled DNA was introduced in the transfer step, with or without DEAE-dextran. Cells were

pelleted at the end of the transfer step and assayed for ^3H -pBR322 binding to the cell pellets. The following features are noteworthy. (a) Little DNA is associated with the cells without DEAE-dextran and without lysis (Table I, experiment 1). (b) The presence of DEAE-dextran in the transfer step (experiment 2) causes some association of ^3H -pBR322 to the cell above background (experiment 1) even without swelling or rupturing the cells. (c) If the cells are swollen and they proceed to rupture in the transfer step without DEAE-dextran present (experiment 3), a small amount of DNA associates with the cells above the background level (experiment 1), perhaps due to DNA trapping within the cells. (d) If the cells are subjected to the standard 9-d osmotic transfer, including a swelling step followed by rupture of the majority of the cells in the transfer step with DEAE-dextran present, nearly all of the DNA is found bound to the cells (experiment 4). (e) Substitution of hypoionic hypotonic lysis for NH_4Cl results in nearly all the DNA binding to the cells (experiment 5), though CAT expression by the cells is 10–20 times less (data not shown) than in cells osmotically lysed in isoionic or hyperionic NH_4Cl (perhaps because of the exposure to low ionic strength). (f) Hypotonic lysis followed by membrane resealing at 37°C for 5 min before the transfer step results in marked reduction of DNA associated with the cells (experiment 6). We infer that both DEAE-dextran and transient contact between the extra- and intracellular spaces during the transfer step markedly increase the association of DNA with the cells.

Optimum DNA and Cell Concentrations for Gene Expression in Osmotic Transfection

Using the standard 9-d osmotic transfer, we varied the DNA concentration in the transfer step. Subsequent CAT expres-

Table I. ^3H -pBR322 Binding to Embryonic Chicken Erythrocytes

Experiment	Pretransfer step treatment				DEAE-dextran in transfer step	DNA bound
	250 mM NH_4Cl	PBS	5 mM NH_4Cl	5 mM NH_4Cl followed by resealing		
1	—	+	—	—	—	0.1 (0)
2	—	+	—	—	+	5.4 (3.2)
3	+	—	—	—	—	3.5 (3.3)
4	+	—	—	—	+	97 (3.5)
5	—	—	+	—	+	99 (0.4)
6	—	—	—	+	+	28 (3.3)

Chicken erythrocytes (5×10^7) from 9-d-old chicken embryos were resuspended in 0.5 ml 250 mM NH_4Cl , PBS, or 5 mM NH_4Cl in a swelling step or substitute (pretransfer step) for 45 min at 22°C with or without 300 $\mu\text{g}/\text{ml}$ DEAE-dextran. In experiment 6, the cells were subjected to a membrane resealing procedure for the last 15 min of this step by adding NH_4Cl to a concentration of 150 mM and incubating at 37°C . The cells from all six experiments were pelleted at 10,500 g for 1 min and resuspended in the standard transfer step solution containing ^3H -pBR DNA (1.7×10^{10} cpm/ μmol) except that DEAE-dextran was withheld in this step in the indicated cases. After 10 min at 37°C , the cells were pelleted at 10,500 g for 1 min. The supernatant was removed and aliquots of the supernatant and pellet were counted for radioactivity in a scintillation counter. The percentage of total radioactivity found in the pellet is the percentage DNA bound to the cells. The Hb release in experiments 3 and 4 were 20, 60, and 80% in the swelling step, transfer step, and total, respectively. Hb release in 5 mM NH_4Cl in experiments 5 and 6 were 99%.

Numbers in parenthesis are standard deviations expressed as percentages.

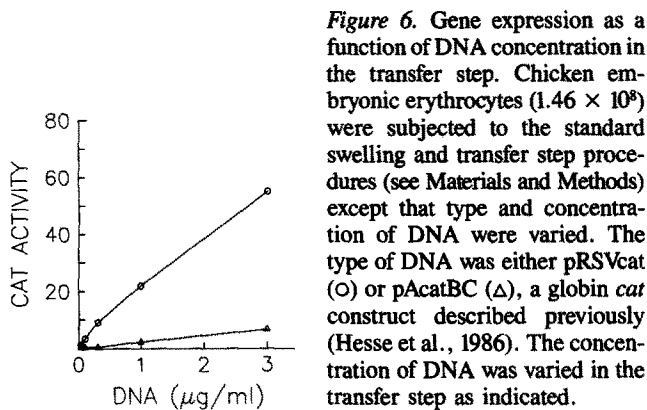


Figure 6. Gene expression as a function of DNA concentration in the transfer step. Chicken embryonic erythrocytes (1.46×10^8) were subjected to the standard swelling and transfer step procedures (see Materials and Methods) except that type and concentration of DNA were varied. The type of DNA was either pRSVcat (○) or pAcatBC (△), a globin *cat* construct described previously (Hesse et al., 1986). The concentration of DNA was varied in the transfer step as indicated.

sion is linear up to 3 µg DNA/ml (Fig. 6) and is proportional to the molar amount of DNA (not shown). Above 3 µg DNA/ml, expression declines (data not shown). Therefore, absolute activity comparisons of different sized expression vectors should be done at equivalent molar amounts of DNA. Expression increases with cell concentration up to 10^8 cells/ml if the cell titration is done using a swelling step incubation time that represents the expression peak (Fig. 7) (see Discussion). The details of the dependence of CAT expression on concentrations of cells or of DNA vary with the choice of conditions. For example, raising the swelling step incubation temperature to 25°C results in an upward shift in the concentration of 9-d embryonic cells corresponding to peak expression, and an increase in the amount of CAT expression observed at the peak (data not shown).

Transcriptional and Translational Biosynthesis in Cells after Osmotic Transfection

Within 24 h after osmotic rupture, transfected cells recover nearly normal transcriptional and translational capability. 4-d embryonic erythrocytes were osmotically transfected

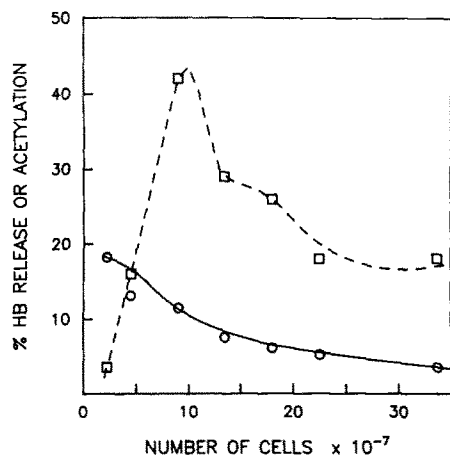


Figure 7. Gene expression and Hb release in swelling step as a function of cell number in transfection. Chicken erythrocytes from 9-d embryos were treated by the standard swelling and transfer procedures (see Materials and Methods) except that the cell number in the procedure was varied as indicated. Percentage Hb release in the swelling step (○) and CAT activity for the specified number of cells (□) were measured. CAT acetylation values shown are 0.33 times their actual values.

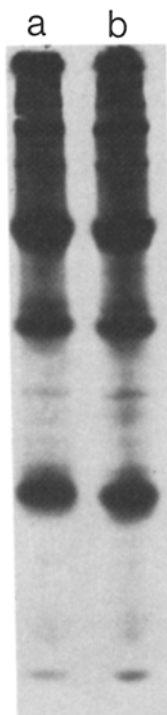


Figure 8. Protein biosynthesis in 4-d embryonic erythrocytes after osmotic transfection. 4-day embryonic erythrocytes (5×10^7 cells) were subjected to a mock osmotic transfection using the standard conditions for 4-day cells (see Materials and Methods) but with nonspecific plasmid DNA (a). Control 4-d cells were not subjected to this procedure (b). Each sample was subsequently incubated for 24 h in RPMI media with [35 S]methionine and without unlabeled methionine. At 24 h, lysates of the cells were prepared by pelleting the cells and resuspending in 100 mM NH_4HCO_3 at 37°C for 5 min. The lysates were run on a polyacrylamide-Triton X-urea gel.

with pRSVcat using the standard 4-d embryonic erythrocyte osmotic transfer (see Materials and Methods). The transfected cells were subsequently incubated with [35 S]methionine in methionine-free medium. Extracts of the labeled cells were prepared after 24 or 48 h. Incorporation of [35 S]methionine into protein was assessed quantitatively by TCA precipitation and qualitatively by running denaturing polyacrylamide gels to observe the representation of incorporated methionine in the major protein species (Fig. 8). At 24 h after transfer, the cells were approximately as active as cells that had not been subjected to the osmotic transfer procedure. This is indicated qualitatively in Fig. 8 by the relative intensity and proportions of protein bands from transfected

Table II. Differential Gene Expression as a Function of Developmental Age of Osmotically Transfected Embryonic Erythrocytes

Age	Acetylation			Ratio of AcatE to Acat	Ratio of AcatE to RSVcat
	Acat	AcatE	RSVcat		
days	%	%	%		
3	0.45	4.3	73	9.5	0.06
4	0.34	8.3	80	24	0.10
5	0.60	24	81	40	0.30
9	0.40	40	76	100	0.53
11	0.66	46	67	70	0.68
12	0.25	17	32	70	0.54
17	0	0.23	0.38	—	0.60

Cells were osmotically transfected with either pRSVcat, pAcat, or pAcatE (see text). The length of the swelling step incubation was chosen for optimal *cat* vector expression for each developmental age (see Figs. 6 and 7, and Materials and Methods). Cell extracts were prepared 48 h after transfection and assayed for CAT. Data are expressed as percentage [14 C]chloramphenicol acetylated in 60 min by 10^8 cells or as a unitless ratio of two percentages.

The average number of cells used in two to five replicate transfections at each age are as follows: 3 d, 4.3×10^7 ; 4 d, 10.5×10^7 ; 5 d, 15.3×10^7 ; 9 d, 11.2×10^7 ; 11 d, 13.9×10^7 ; 12 d, 11.1×10^7 ; and 17 d, 11.9×10^7 .

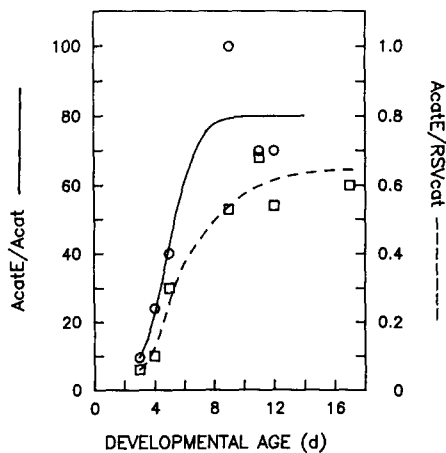


Figure 9. Differential gene expression by osmotically transfected embryonic erythrocytes as a function of developmental age. Embryonic erythrocytes were osmotically transfected with pRSVcat, pAcotE, or pAcat using the standard osmotic transfection conditions. The length of the swelling step incubation was chosen for optimal pRSVcat vector expression for each developmental age (see Materials and Methods and Figs. 6 and 7). Cell extracts for CAT assay were prepared 48 h after transfection. The ratio of CAT expression of pAcotE to pAcat (○, —) and the ratio of pAcotE to pRSVcat (□, - - -) are plotted as a function of the developmental age of the osmotically transfected erythrocytes.

and untransfected cells. Quantitatively, 1.3×10^6 cpm was incorporated into both the transfected cell lysate and the untransfected control cell lysate. The 48-h time point gave the same gel results as the 24-h point. Similar results were obtained for 5-, 9-, and 12-d cells or when [^3H]leucine incorporation was measured (data not shown). In other experiments, 9-d cells were labeled for 5 h after osmotic transfer with [^{35}S]methionine. At this shorter incubation time, methionine incorporation was already $\sim 50\%$ of the levels of unshocked control cells.

Further evidence of the biosynthetic capabilities of osmotically transfected cells is revealed by transient expression. CAT expression in 4-d embryonic erythrocytes is detectable by enzymatic assay 24 h after osmotic transfer, although accumulated CAT levels at this time are only one-third their 48 h levels (data not shown). Because expression of transfected vectors requires mRNA and protein biosynthesis, transfected cells must be capable of transcription and translation within 24 h after osmotic transfer.

Fidelity of Transcriptional Specificity after Osmotic Transfection

We were interested in determining whether gene expression in cells subjected to the seemingly harsh conditions of osmotic transfection continued according to the cellular developmental program. pRSVcat or the globin-cat expression vectors, pAcat or pAcotE (Hesse et al., 1986), were osmotically transfected into 3-, 4-, 5-, 9-, 11-, 12-, or 17-d embryonic erythrocytes using the respective swelling step incubation times appropriate for peak expression at each age (see Materials and Methods). The plasmid pAcat contains the 5' flanking region of the chicken β^A -globin gene placed upstream of the *cat* structural gene. The plasmid pAcotE is identical except for the addition just downstream of the *cat*

structural gene of another fragment from the β^A -globin gene. This fragment, originally located 110–588 bp on the 3' side of the β^A -globin polyadenylation signal in the endogenous β^A -globin gene, contains a sequence with the properties of an enhancer (Hesse et al., 1986). In 3- and 4-d embryonic erythrocytes, pAcotE directs ~ 10 -fold more expression of CAT than pAcat (Table II; Fig. 9). In 5-, 9-, and 12-d erythrocytes, pAcotE directs 40-, 100-, and 70-fold more expression than pAcat, consistent with our earlier results at these three ages. In 11- and 17-d erythrocytes, results are obtained similar to those in 12-d cells. In 12- and 17-d erythrocytes, the absolute levels of transcription of all three plasmids was lower. This is consistent with the overall decline in transcriptional activity known previously to begin at 12 d of avian erythroid development (Landes et al., 1982).

The ratio of CAT expression pAcotE/pAcat as a function of developmental age indicates that the enhancer fragment confers on the β -globin promoter the capacity for developmental activation (Fig. 9). The time course of this developmental activation agrees very well with studies after endogenous β -globin protein and mRNA levels as a function of developmental age (Bruns and Ingram, 1973; Brown and Ingram, 1974; Landes et al., 1982). Equally important, this agreement indicates that these transfected embryonic erythrocytes adhere to their developmental program.

Discussion

Proposed Mechanism of Osmotic Transfection

Our procedure involves swelling cells in NH_4Cl immediately before DEAE-dextran-DNA treatment (Fig. 10). Some cells rupture in this swelling step, but most apparently only approach their maximum volume. The intracellular osmotic pressure at this point has necessarily increased (see below). The cells are pelleted and resuspended in the iso-osmotic transfer solution. Due to the tonicity difference between the interior (approaching 500 mosM) and the exterior (290 mosM), most of the remaining unlysed cells rupture quickly after resuspension in the transfer step solution.

DNA enters the cells, perhaps through the transient hole created by hemolysis. The hole closes within seconds (Seeman, 1967) to the size of a few phospholipids (radius < 2 nm under isoionic conditions; Lieber and Steck, 1982a, b) and is apparently repaired. Cellular transcription and translation resume to near normal levels within hours, and transferred DNA molecules are expressed in accord with the cell differentiation program. Individual aspects of this mechanism are described below.

Cell Volume and NH_4Cl

The mechanism by which NH_4Cl solutions swell erythroid cells is well described (Jacobs and Stewart, 1947; Knauf, 1979). NH_4^+ is in equilibrium with NH_3 , which diffuses freely across membranes. In general, eukaryotic cells are impermeable to anions and preservation of electroneutrality prevents any significant equilibration of NH_4^+ across the membrane without its counterion. Therefore, when cells are placed in 150 mM NH_4Cl , the intracellular and extracellular tonicities are equal at roughly 300 mosM, and the cells maintain a stable volume. However, erythroid cells are an exception to this because they possess a membrane anion trans-

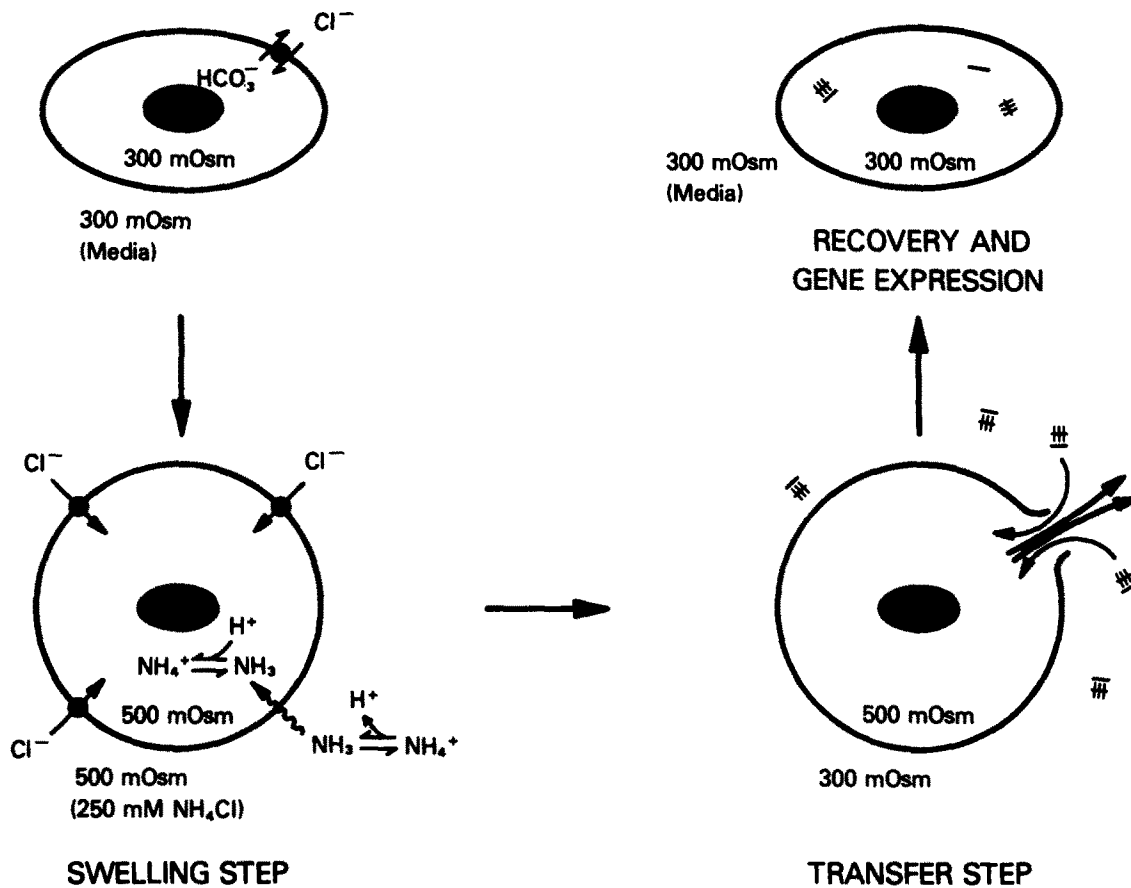


Figure 10. Proposed mechanism of osmotic transfection. The line (—) represents DNA and the crossed line (++) represents DEAE-dextran.

port protein, Band 3, which equilibrates several anionic species, including Cl^- , across red cell membranes for the physiologic purpose of the $\text{Cl}^-/\text{HCO}_3^-$ shift. Thus, both NH_4^+ and Cl^- equilibrate across the red cell membrane. As the intracellular concentration of NH_4Cl increases approaching the extracellular concentration, the external medium no longer exerts sufficient osmotic pressure to balance that of the intracellular impermeable molecules plus the acquired NH_4^+ and Cl^- . Therefore, the cells swell. If unchecked, this process results in osmotic rupture of the membrane quickly after the cells become spherocytic.

The time course of swelling and lysis can be delayed by using 250 mM NH_4Cl . This hypertonic solution initially causes rapid shrinkage of the cells because of diffusion of water across the membrane. The cells shrink until their internal osmotic pressure matches that of the external solution of 500 mosM then swell as the NH_4^+ and Cl^- progressively equilibrate, a process that takes longer with an external NH_4Cl concentration of 250 than 150 mM. The combined effect of this longer uptake interval and the initially shrunken state results in a much longer time lag in the swelling step before lysis. The osmotic pressure in the spherocytic erythrocyte just before lysis is 500 mosM. Upon resuspension in the transfer step solution, most of the already swollen cells rupture in the 290 mosM transfer solution.

It should be noted that the transfer step solution is 0.66 strength L-15 media and 50 mM Tris·Cl. The Tris cation can

deprotonate and diffuse across the bilayer just as NH_3 does, making Tris·Cl, like NH_4Cl , a permeable salt. Therefore, the transfer step solution is effectively hypotonic with an effective tonicity of only 190 mosM. Consequently, the hypotonic transfer step incubation alone is sufficient to achieve significant Hb release and DNA transfer in the somewhat more fragile and, therefore, sometimes swollen erythrocytes of the younger embryonic ages (3 and 4 d). This may explain why some subsequent expression can be seen even at zero swelling time (Fig. 4 D). In some preparations of 4-d embryonic erythrocytes, the amount of expression seen at zero swelling time is comparable to that observed in the NH_4Cl wash (data not shown).

Active Cellular Species in Osmotic Transfection

Is osmotically enhanced gene expression mediated by the unlysed, swollen erythrocyte or the ruptured erythrocyte? The time courses of expression and Hb release (Figs. 2 and 4) indicate that DNA expression, in most cases, is greater than that achieved without swelling (zero time point) even when 96–100% of the cells are ruptured. Furthermore, if the swollen, unruptured erythrocyte were the cellular species active in transfection, then the peak of expression should occur well before the 50% total Hb release point in the simplest model. In fact, it is near the 85% total Hb release point. Commonly, after the transfer step, the cell pellets contain no

red, but only white or pink cells, indicating that no obvious portion of the population escaped the transient osmotic poration. When we transfect 9-d embryonic erythrocytes using conditions that merely swell them, but do not significantly rupture them, the expression is much lower. These observations strongly indicate that the transiently ruptured cell is the active species.

Is it the cell that ruptures in the swelling step or in the transfer step that is active in expression of transfected DNA? In some time courses, there is significant expression when lysis occurs only in the transfer step (Fig. 2, C and D), and not in the swelling step. Therefore, cells rupturing in the transfer step are active. Some of the cells that rupture in the swelling step may also be active, but our results do not address this point. For reasons discussed in an earlier paper (Hesse et al., 1986) we do not think that a nonerythroid contaminant cell population can be responsible for the observed CAT expression.

Role of DEAE-Dextran in Osmotic Transfection

DNA binds to and pellets with ruptured avian erythrocytes from 9-d embryos when in solution with DEAE-dextran (Table I). Without DEAE-dextran, unruptured cells bind only 0.1% of the DNA, and the amount of DNA associated with ruptured cells is roughly what one would predict based on simple equilibration between the intracellular and extracellular space (~4%). With DEAE-dextran, 5% of the DNA pellets with unruptured cells. DNA binding increases to >95% after rupture, and correlates with a subsequent 40-fold increase in *cat* gene expression. It seems likely that the increase in expression is a result of increased DNA within the cell as opposed to a general stimulation of transcription or translation.

Why does DEAE-dextran boost DNA association with ruptured cells 20-fold over that observed with unlysed cells? Resealing ruptured cells before the transfer step reduces this association, toward that of the unlysed state (Table I). The increased association thus requires transient continuity between the intracellular space and the extracellular DNA during the transfer step. The amount of DNA that binds to the transiently patent erythrocytes is 25-fold greater than that expected based on simple diffusional equilibration. This suggests that under these circumstances, DEAE-dextran causes binding of DNA predominantly to the intracellular region rather than to the outer surface of the membrane.

Relationship of Osmotic Transfection to Other Methods of Macromolecular Transfer

In our early comparisons of transfection methods, we observed that even the conventional DEAE-dextran transfection method is associated with some hemoglobin release above background (Fig. 1). In additional studies, we incubated cells in media with DEAE-dextran alone, with DNA alone, or both. We found that with DEAE-dextran 2–5% of the hemoglobin is released, whereas DNA alone causes no release above background (data not shown). DNA plus DEAE-dextran causes 5–10% hemoglobin release. Therefore, DEAE-dextran is mildly lytic to erythrocytes and the presence of DNA increases this effect.

Is DEAE-dextran transfection a low efficiency version of osmotic transfection? We find a good correlation between

percentage hemoglobin release during the transfer step and subsequent vector expression (Figs. 1, 2, 4, and 5). For avian erythrocytes, it appears that conventional DEAE-dextran transfection may act via transient cell rupture followed by DNA association. Osmotic transfer increases the efficiency of the lysis step and uses the DEAE-dextran primarily for binding the DNA to the cell. Similar studies comparing conventional DEAE-dextran and an adaptation of this osmotic transfer method to murine lymphocytes and several other hematopoietic lineages support these inferences (Lieber et al., 1987).

The use of glycerol shock to boost calcium phosphate and DEAE-dextran transfection in other cells appears to serve a function similar to that of NH_4Cl in these studies. Biological membranes are permeable to glycerol. Exposure to hypertonic concentrations as described (Parker and Stark, 1979) presumably results in initial shrinkage of the cells, followed by glycerol equilibration and swelling, just as in the 250 mM NH_4Cl swelling step here. Transfer back to isotonic media creates a large osmotic pressure drop between the now hypertonic cytoplasm and the media. Surface-bound DNA may now enter the cell.

There are several other methods that may share significant features with osmotic transfection. The method of scrape loading adherent cells is known to rupture them transiently but allows macromolecules to enter with considerable cell viability (McNeil et al., 1984). The use of $0.66\times$ media in the pinocytotic vesicle transfer procedure (Okada and Rechsteiner, 1982) is not unlike our DNA transfers using media dilutions (Fig. 1). Finally, electroporation is an alternative method of introducing transient membrane holes in cells (Neumann et al., 1982; Potter et al., 1984). At least in the case of nucleated erythrocytes, the advantage of osmotic transfer is that each cell contains only a single small rupture site, viability and biosynthesis appear good, reproducibility is excellent, primary cells along a developmental progression can be used, and no special equipment is required.

We have successfully introduced a nuclease into the avian erythrocytes using the osmotic transfer procedure described here (Lieber, M. R., J. E. Hesse, G. Felsenfeld, unpublished data). Thus, applications of this osmotic transfer method may be extended to include the introduction of nuclear regulatory factors into primary cell populations.

Basis for the DNA Concentration and Cell Density Optima

As mentioned above, DNA increases the lytic effect of DEAE-dextran. It appears that higher DNA concentrations in the transfer step act to increase the Hb release in that step and lead to excessive lysis (not shown), perhaps accounting for the decrease in gene expression observed at these DNA concentrations (Fig. 6). It is possible that shorter swelling step intervals would permit the use of higher DNA concentrations in the transfer step.

The eventual decline in gene expression per cell at high cell density (Fig. 7) may be due to the inhibition of Hb release at high extracellular protein concentrations. In general, osmotic lysis is an all-or-none process. However, when the extracellular protein concentration reaches 5–10 mg/ml, a solvent-exclusion effect appears to inhibit Hb efflux so that partial Hb release from individual cells occurs (Seeman,

1973). At high cell densities, the Hb contributed by the initial rupturing cells may inhibit the release by the cells lysing subsequently. In these later lysing cells, less Hb exits, and there may also be less opportunity for extracellular macromolecular entry.

Fidelity of β -Globin Transcriptional Activation in Osmotically Transfected Erythrocytes

Despite losing most of their cytoplasmic protein, osmotically transfected embryonic erythrocytes recover 50% of their protein biosynthetic capacity within 5 h of transfer and achieve normal translational levels of the observed proteins within 20–25 h. However, even given this remarkable cell recovery, osmotic transfection would not be very useful if the procedure disrupted the normal cellular developmental program. We have recently described experiments suggesting that such specificity might be retained. In those experiments, 9-d-old embryonic erythrocytes were transfected with plasmids in which the promoter region of the adult β -globin gene is fused to the 5' end of the *cat* gene, while a sequence from the 3' flanking region of the β -globin gene is fused at the 3' end of the *cat* gene. The 3' globin sequence was shown to have the properties of an enhancer in these red cells, but not in chick fibroblasts, suggesting that the red cells retained factors that specifically regulated β -globin gene expression.

In this paper, we show that the effect of the enhancer on CAT expression is markedly reduced when this plasmid is introduced into 3- or 4-d-old embryonic erythrocytes, in which the endogenous chicken adult β -globin gene is not detectably expressed. Furthermore, when the plasmid is introduced into successively older embryonic cells from 5- to 17-d-old embryos, the stimulatory effect of the enhancer on CAT expression increases in a manner roughly consistent with the activity of the endogenous globin genes (Landes et al., 1982). This suggests that the 3' putative enhancer sequence, perhaps in combination with the β -globin promoter, confers developmental stage specificity. It also supports the idea that at all stages of development, osmotic transfection does not destroy or alter the developmental program of these cells.

Concluding Remarks

The mechanistic details for osmotic transfection have been described for the major developmental phases of chick erythroid differentiation. In elucidating these mechanisms, we have observed that the amount of CAT expression, although quite reproducible for any given set of well controlled conditions, depends rather sensitively on NH_4Cl , DNA, and cell concentrations. Investigators using this system are advised to construct their own standard curves for the swelling step, and DNA and cell concentrations.

The results we have described make primary nucleated erythrocytes a relatively well-understood system of non-neoplastic cells in which to do transient expression studies with efficient DNA transfer and high levels of subsequent expression. The method described here was devised in order to study the developmental regulation of chicken globin gene expression. In an earlier report (Hesse et al., 1986) we used it to identify a tissue-specific enhancer element at the 3' end of the adult β -globin gene. We have shown here that osmotic transfection is applicable to 3-d and older embryonic red

cells. This covers nearly the entire developmental time course of interest for globin gene expression. We have documented the developmental fidelity with which a transfected β -globin gene construct bearing the 3' β -globin enhancer is expressed in these cells. All of these results show that osmotic transfection permits the use of avian, and probably other nucleated red cells, for the analysis of the specific signals associated with red cell development.

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