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 NH₄Cl 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 \(\beta\)-globin promoter and the 3' \(\beta\)-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.

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 Na₂HPO₄ per liter, 0.12 g of anhydrous KH₂PO₄ 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 ~10⁷ 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 (10⁶ 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 NH₄Cl (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 NH₄Cl 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 NH₄Cl 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; 5 x 10⁶ D] per ml, and 1-3 µg of DNA per ml), which was prepared 3-120 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, and 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 NH₄HCO₃ 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) 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 [³⁵C]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 x 10⁶ 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 NH₄HCO₃ (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 (Rommann, 1960), the cell number was calculated from the absorbance. Cell number equals the optical density (412 nm) times 8.52 x 10⁶ for 3-d RBC, 6.52 x 10⁶ for 4-d RBC, 5.34 x 10⁶ for 5-d RBC, 7.70 x 10⁶ for 10-d RBC, and 9.31 x 10⁶ for 4-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 toxicity 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 toxicity of 140 mosM (Fig. 1).
We found that we could swell the cells while maintaining a physiologic ionic strength, by using NH₄Cl in a swelling step administered immediately before DEAE-dextran (transfer step) treatment (see Discussion). Cells were swollen by incubation in 150 mM NH₄Cl, 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 NH₄Cl solutions (200 or 250 mM) or in mixtures of NH₄Cl 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 NH₄Cl 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 (~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 μm.

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 ($V_o$) and lytic cell volume ($V_L$) relative to $t^*$, the time for half of the cells to lyse, is given by the equation: $V_o/2 \left( \frac{V_L}{V_o} \right)^2 - 1 = k t^*$, where $k$ is the composite permeability coefficient for $\text{NH}_4^+$ and $\text{Cl}^-$ and reflects the mode by which $\text{NH}_4\text{Cl}$ enters

![Graphs showing Hb release and gene expression as a function of swelling step time for embryonic erythrocytes at different developmental ages.](image)

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 NH4Cl 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 (△) and transfer step (□) were measured spectrophotometrically (see Materials and Methods). The sum of Hb release in these two steps is the total Hb release (○). The cells were then pelleted, washed once in L15, and resuspended in media (see Materials and Methods). At 48 h after transfection, the cells were harvested and assayed for CAT (○) as described in Materials and Methods. Chloramphenicol acetylation values shown are normalized to 1 × 10^8 cells, and the activity values in Care 0.33 times their actual values. Transfer step Hb release was not measured for 5 d cells.
lates developmental age and cell volume. The ratio of cell volume per unit surface area and is relatively constant from embryonic days 4 to 18 (Chart, 1977). The ratio of cell volume per unit surface area and is relatively constant from embryonic days 4 to 18 (Chart, 1977).

The ratio expression (n, - - - -) in Fig. 4 (,4, B, C, and D) are plotted as a function of cell volume (Romanoff, 1960). The solid line also relates developmental age and cell volume.

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 ^H-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 ^H-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 ^H-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 NH4Cl 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 NH4Cl (perhaps because of the exposure to low ionic strength). (f) Hypotonic lysis followed by membrane rescaling 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-
Figure 6. Gene expression as a function of DNA concentration in the transfer step. Chicken embryonic erythrocytes (1.46 x 10^7) 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 (C) or pACatBC (Δ), a globin cat construct described previously (Hesse et al., 1986). The concentration of DNA was varied in the transfer step as indicated.

Figure 8. Protein biosynthesis in 4-d embryonic erythrocytes after osmotic transfection. 4-day embryonic erythrocytes (5 x 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 [35S]methionine and without unlabeled methionine. At 24 h, lysates of the cells were prepared by pelleting the cells and resuspending in 100 mM NH4HCO3 at 37°C for 5 min. The lysates were run on a polyacrylamide-Triton X-urea gel.

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 with pRSVcat using the standard 4-d embryonic erythrocyte osmotic transfer (see Materials and Methods). The transfected cells were subsequently incubated with [35S]methionine in methionine-free medium. Extracts of the labeled cells were prepared after 24 or 48 h. Incorporation of [35S]methionine into protein was assessed qualitatively 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

<table>
<thead>
<tr>
<th>Age</th>
<th>Acat</th>
<th>AcatE</th>
<th>RSVcat</th>
<th>Ratio of AcatE to Acat</th>
<th>Ratio of AcatE to RSVcat</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
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<td>3</td>
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<td>73</td>
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<td>0.06</td>
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<td>0.10</td>
</tr>
<tr>
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<td>0.60</td>
<td>24</td>
<td>81</td>
<td>40</td>
<td>0.30</td>
</tr>
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</tr>
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<td>0.38</td>
<td></td>
<td>0.60</td>
</tr>
</tbody>
</table>

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 [%] chloramphenicol acetylated in 60 min by 10^6 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 x 10^7; 4 d, 10.5 x 10^7; 5 d, 15.3 x 10^7; 9 d, 11.2 x 10^7; 11 d, 13.9 x 10^7; 12 d, 11.1 x 10^7; and 17 d, 11.9 x 10^7.

Expression 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).

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 step procedures (see Materials and Methods) except that type and concentration of DNA were varied. The type of DNA was either pRSVcat (C) or pACatBC (Δ), a globin cat construct described previously (Hesse et al., 1986). The concentration of DNA was varied in the transfer step as indicated.

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bryonic erythrocytes were osmotically transfected with pRSVcat, Figure 9. 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 pAcatE to pAcat (○, ——) and the ratio of pAcatE to pRSVcat (□, - - - -) are plotted as a function of the developmental age of the osmotically transfected erythrocytes.

Discussion

Proposed Mechanism of Osmotic Transfection

Our procedure involves swelling cells in NH4Cl 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 toxicity 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 isionic 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 NH4Cl

The mechanism by which NH4Cl solutions swell erythroid cells is well described (Jacobs and Stewart, 1947; Knauf, 1979). NH4+ is in equilibrium with NH3, which diffuses freely across membranes. In general, eukaryotic cells are impermeable to anions and preservation of electroneutrality prevents any significant equilibration of NH4+ across the membrane without its counterion. Therefore, when cells are placed in 150 mM NH4Cl, 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 (+++)...
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 NH4Cl 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 NH4Cl 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 (McNeill et al., 1984). The use of 0.66× media in the pinocytic vesicle transfer procedure (Okada and Rechsteiner, 1982) is not unlike our DNA transfers using media dilutions (Fig. 1). Finally, electroproporation 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 transfection 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,
expression. The method described here was devised in order to study the developmental regulation of chicken 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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