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Changes in cell surface charge and transmembrane potential accompanying neoplastic transformation of rat kidney cells

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Free flow electrophoresis measurements have been used to determine the surface charge density of normal rat kidney (NRK) cells and a clone of NRK, designated as 6m2, that exhibit a transformed phenotype at 33° C and a non-transformed phenotype at 39° C. A clone of 6m2, designated 54-5A4, which is transformed at both 33° C and 39° C was also studied. A surface charge density of $-1.42\ \mu C/cm^2$ was obtained for the NRK and non-transformed 6m2 cells at 39° C, whereas at 33° C values of -1.85 and $-1.78\ \mu C/cm^2$ were determined for the transformed 6m2 and 54-5A4 cells, respectively. It was found that 72% of the increased charge that appeared on the transformed 6m2 cells compared with the non-transformed 6m2 cells was RNAase sensitive. The time-dependent decrease in surface charge that accompanied the shift of the 6m2 cells from their transformed to non-transformed state was found to mirror the increase in transmembrane potential previously reported using a fluorescent dye technique, and was also comparable to the reported temporal changes in their morphology and virally-coded protein content.

Introduction

It has generally been observed that the transmembrane potential difference (E_m) and the cell membrane surface charge density (σ) alter when mammalian cells undergo neoplastic transformation. With relatively few exceptions [1–3], E_m has been reported to be lower [4–14] in transformed cells compared with their non-transformed counterparts. Also, σ assumes a more negative value in transformed cells [15,16] and some cancerous tissues have been found to be more electronegative [17–19] and others more electropositive [19] than non-transformed tissues. Although data have been obtained [20–24] which support the original proposal by Cone [25] that E_m is a regulator of mitosis for both non-transformed and neoplastic cells, the finding [1–3] that some transformed cells do not have a lower E_m value than their non-transformed counterparts indicates, contrary to the suggestion by Cone and Tongier [20], that transformed cells do not necessarily possess lower E_m values that reflect their proliferative capacity. Also, Simon-Reuss et al. [26] were unable to find a general relationship between cell surface charge and the type of cell, site of origin, rate of growth or malignancy of the parent tissue. Changes in such electrical parameters as the E_m and σ of a

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cell membrane certainly reflect fundamental changes in the physiological state of that cell, but it would appear that more work is required before a full understanding of this can be achieved.

Values for E_m are often determined by inserting a microelectrode into the cell interior and measuring the potential difference between this electrode and another one situated in the extracellular medium. This is the method adopted in most of the E_m measurements cited above. In their measurements of the relative changes in E_m that accompany cell transformation, Lai et al. [13] employed a fluorometric technique using a cyanine dye. Although measurement of the dye fluorescence provides only an indication of relative changes in E_m and not an absolute value [27,28], in our opinion this method is capable of determining more clearly changes in the total electrical potential at the inner plasma membrane surface (i.e. $E_m + \Phi_i$, see Fig. 3) than is achieved using an invasive microelectrode.

In this paper measurements are reported of the changes in cell surface charge that accompany alterations in phenotype of the same cell culture system used in the fluorescence studies [13]. The cells studied were normal rat kidney cells infected with the temperature-sensitive transformation mutant of Moloney murine sarcoma virus (ts110) that yields a clone of cells (6m2) exhibiting a transformed phenotype at 33°C and a non-transformed phenotype at 39°C [29]. A normal rat kidney cell line (NRK) and a clone of 6m2, designated 54-5A4, which is transformed at both 33°C and 39°C [30], were used as controls. We show here that the 6m2 cells of transformed phenotype have a more positive inner membrane potential (a result derived from the previous work [13]) and a more negative surface charge density than those of the non-transformed phenotype.

As part of these studies we have also investigated to what extent the negative charges on the cells are RNAase-sensitive as has been found for various ascites and sarcoma cells [31]. The non-transformed 6m2 cells had no RNAase-sensitive surface charge, but for the transformed 6m2 cells it was found that around 17% of their total surface charge, equivalent to 72% of the difference between the non-transformed and transformed values, was RNAase sensitive.

Materials and Methods

(a) Cell lines

The 6m2 cells were originally isolated by Blair, Hull and Finch [29] and were maintainted at either 33°C (transformed phenotype) or 39°C (non-transformed phenotype). NRK cells, obtained from Dr. J. Knesek of UT M.D. Anderson Hospital, and 54-5A4 cells [30] were maintained at 37°C. All cells were cultured for at least two days at the appropriate temperature in McCoy's 5a medium supplemented with 15% fetal calf serum. Confluent cells were harvested by scraping the 6m2 and 54-5A4 cells and trypsinizing the NRK cells [13]. After harvesting, the cells were washed and resuspended (approx. $5 \cdot 10^5$ cells/ml) in Dulbecco's phosphate-buffered saline (pH 7.2) at the appropriate measurement temperature. This suspension was then used for the electrophoretic mobility measurements.

(b) Electrophoretic mobility measurements

Measurements were made using a Rank Brothers (Bottisham, Cambridge, U.K.) Mk III electrophoresis chamber of the type described by Sutherland and Pritchard [32] in combination with a Cam-Apparatus (Cambridge, U.K.) optical assembly and a constant temperature bath which maintained the temperature to within 0.2°C during measurements. The electrophoretic mobility was determined by measuring the time taken for the cells to travel a distance of 48 μm at the 'stationary layer' [32] under the influence of an electric field produced by a constant current source of 2 mA. The magnitude of the field was calculated from the known conductivity of the solution. In each experiment at least forty cells were measured in the forward and reverse field directions. The timing measurements were directly interfaced to a computer which calculated the mean mobility, standard deviation, zeta potential, and surface charge density. The equipment was tested with three-times washed human erythrocytes, which were found to have an electrophoretic mobility of $-1.08\ \mu \cdot s^{-1} \cdot V^{-1} \cdot cm$ in 150 mM NaCl at 25°C and pH 7.2, in good agreement with the established literature value [33].

If the electrophoretic mobility measurements were to be made at a temperature other than that

at which the cells had been cultured, the cells were transferred to an incubator maintained at the mobility measurement temperature for a minimum of 5 h before harvesting. In order to determine the time dependency of the mobility of transformed 6m2 cells after being shifted from 33°C to the temperature at which they revert to their non-transformed phenotype (39°C), the cells were either transferred from the 33°C to the 39°C incubator for set durations before harvesting and mobility measurement, or else were harvested at 33°C and incubated at 39°C in phosphate-buffered saline (pH 7.2) for short periods before measurement. This enabled changes that occurred within the first thirty minutes after the cells were shifted from 33°C to 39°C, as well as longer term changes, to be determined.

(c) RNAase treatment

The procedure used for the ribonuclease (RNAase) treatment of cells was that of Mayhew and Weiss [34]. Approx. 10^7 6m2 cells were harvested and suspended in 1 ml of sterile Hank's balanced salt solution (pH 7.2) at the appropriate temperature. To this cell suspension either 1 ml of the Hank's solution (pH 7.2) was added to provide a control sample, or 1 ml of the Hank's solution (pH 7.2) containing 0.1mg of RNAase was added to provide the 'treated' sample. Either crystalline, salt-free, RNAase (Worthington Biochemical Sales Company, New Jersey, U.S.A.), or essentially protease free RNAase (Type X-A, Sigma) was used. The control and RNAase-treated samples were incubated for 40 min at 33°C or 30 min at 39°C, depending on the transformation state of the cells. Different incubation periods were used to minimize errors that might have resulted from any variation in the enzymic reaction rate with temperature. After incubation, the cells were centrifuged from the sterile Hank's balanced salt solution (pH 7.2) and suspended in Dulbecco's phosphate-buffered saline (pH 7.2) for the electrophoretic mobility measurement at either 33°C or 39°C.

Results

Table I gives the results obtained for the electrophoretic mobility as a function of temperature for the various cells studied, including human erythrocytes. From Table I it can be seen that whereas there was a rise in magnitude of the mobility as the temperature was increased from 33°C to 39°C for the erythrocytes, NRK and 54-5A4 cells, the 6m2 cells exhibited a fall for the same temperature shift. As a control experiment 6m2 and 54-5A4 cells were harvested by the same trypsinization process used for the NRK cells and this was found to produce no significant difference in the cell mobility values compared with those given in Table I. This indicated that the trypsinization procedure did not influence the cell mobility.

TABLE I

VALUES OF THE ELECTROPHORETIC MOBILITY μ OBTAINED AT THE VARIOUS MEASUREMENT TEMPERATURES

Cells	$\mu \pm 0.06$ ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$)		
	33°C	37°C	39°C
Human erythrocytes	−1.21	−1.33	−1.40
NRK	−1.26	−1.40	−1.47
54-5A4	−1.59	−1.75	−1.82
6m2	−1.73	−1.62	−1.46

The mobility of a cell is a function not only of its surface electrical properties but also of the physico-chemical properties, such as the electrical polarizability and highly temperature-dependent viscosity, of the supporting electrolyte. The electrical differences between the various cell types can be more clearly compared if the so-called zeta potential, rather than the mobility, is considered. The zeta potential, ζ, corresponds to the potential at the hydrodynamic plane of shear a small distance beyond the cell surface and can be derived from the electrophoretic mobility μ using the Helmholtz-Smoluchowski equation [35]

$$\zeta = \eta\mu/\varepsilon_o\varepsilon_r \quad (1)$$

where ε_o is the permittivity of free space and η and ε_r represent the viscosity and the real part of the relative permittivity of the suspending electrolyte, respectively. For aqueous solutions of low ionic strengths, of the order of those used in this work, the physical constants [36] for pure water

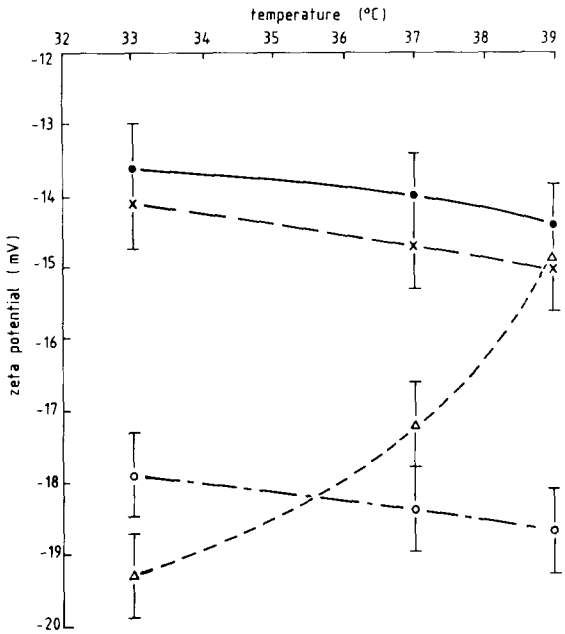

Fig. 1. The temperature variation of the zeta potential measured in Dulbecco's phosphate-buffered saline (pH 7.2) for human erythrocyte (●), NRK (×), 54-5A4 (○) and 6m2 (Δ) cells.

can be used without serious error. A limitation of Eqn. 1 is that changes in viscous drag (and hence mobility) associated with morphological changes of a cell cannot be taken into account. Although, along with other workers in this field, we consider that this does not seriously influence the measurements, this limitation should not in general be completely overlooked.

Fig. 1 shows how the zeta potential, derived from Eqn. 1, varied for the cells, and it can be seen that for the erythrocytes, NRK and 54-5A4 cells the zeta potential became more negative by 4 to 6% as the temperature was increased from 33°C to 39°C, whereas for the 6m2 cells the zeta potential became more positive by 23%. The temperature dependence of the zeta potential for the 54-5A4 cells was similar to that observed for the erythrocytes and NRK cells, but was, significantly, more negative in value. The results obtained for 6m2 cells at 33°C and 54-5A4 cells indicate that the transformed cells exhibit a more negative surface charge than their non-transformed counterparts.

The time dependence of the zeta potential of the 6m2 cells after their temperature had been

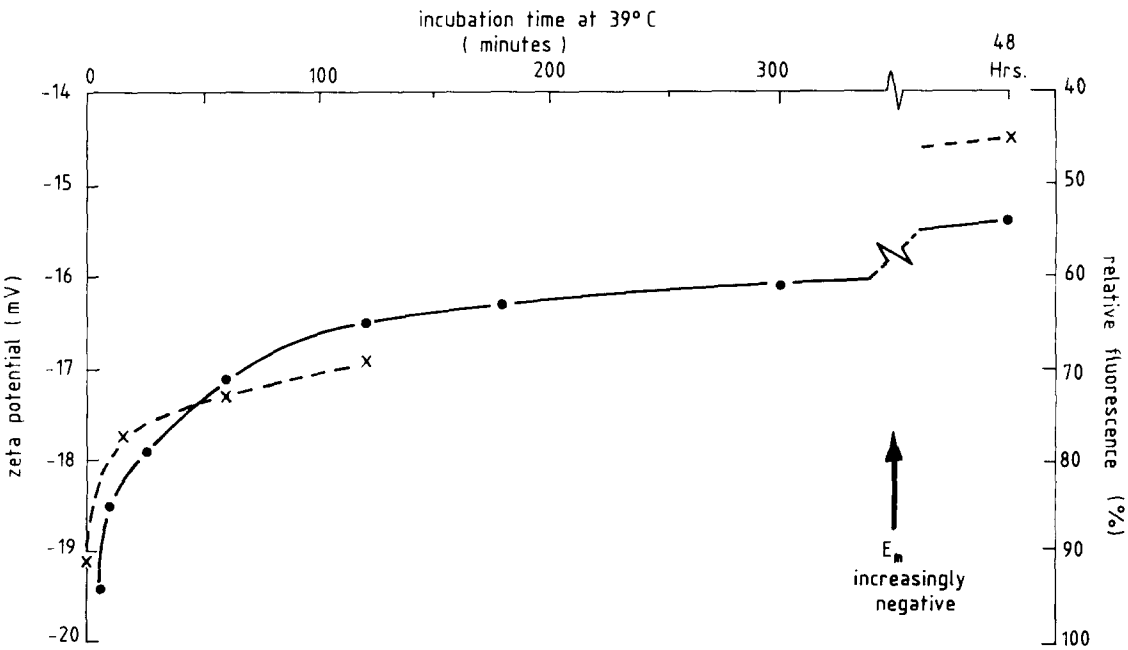

Fig. 2. Variation in relative fluorescence [13] and zeta potential as a function of incubation time at 39°C for 6m2 cells cultured at 33°C. (●, zeta potential; ×, relative fluorescence).

shifted from 33° C to 39° C is shown in Fig. 2. Also included in this figure is the fluorescence data of Lai et al. [13] obtained for 6m2 cells that had experienced the same temperature shift. This data shows that the transformed 6m2 cells have a more negative zeta potential and a more positive internal potential than 6m2 cells exhibiting a non-transformed phenotype. Furthermore, the greatest changes in zeta potential and internal potential occur within 1 h of the cells having their temperature increased from 33° C to 39° C. The fluorescence changes that occurred 15 minutes after the 33° C to 39° C shift were found to be approximately 10-times larger for the 6m2 than for the NRK and 54-5A4 cells [13]. This parallels the relative changes in the zeta potentials shown in Fig. 1.

Table II summarizes the effects of RNAase-treatment of the 6m2 cells. As no significant difference was found in using either the salt free (Worthington) or protease free (Sigma) RNAase, the averages of 3 sets of results are shown in Table II. Whereas RNAase treatment had no effect on the 6m2 cells at 39° C (non-transformed phenotype), a reduction of 17% in the zeta potential was observed for the 6m2 cells in their transformed state at 33° C. This result suggests that the transformed cells possess anionic RNAase-sensitive groups on their surfaces. The absence of an effect for the non-transformed 6m2 cells indicates firstly, that the positively charged RNAase is not adsorbed onto cell surfaces and secondly, that there are no RNAase-sensitive groups originating either indigenously or from the adventitious binding of RNA fragments from cellular debris.

TABLE II

EFFECT OF RNAase TREATMENT ON THE ELECTROPHORETIC MOBILITY μ AND ZETA POTENTIAL ζ OF 6m2 CELLS EXHIBITING A NON-TRANSFORMED (39° C) OR TRANSFORMED PHENOTYPE (33° C)

Measurement temp. (° C)	Treatment	$\mu \pm 0.06$ ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm$)	$\zeta \pm 0.6$ (mV)
39	Control	−1.46	−14.9
39	RNAase	−1.48	−15.1
33	Control	−1.70	−19.0
33	RNAase	−1.40	−15.7

Discussion

In this paper two different types of measurements of the electrical properties of cell membranes have been described, namely surface potential measurements by free flow electrophoresis and transmembrane potential measurements using a fluorescent dye technique. It will be of value to relate these two sets of results, especially in terms of the effects observed on neoplastic transformation of the 6m2 cells.

The electrophoretic mobility results have been interpreted in terms of the zeta potential ζ, which is related to the potential Φ_o existing at the external surface of the cell membrane. No theory has been developed which precisely relates the zeta potential to the actual surface potential, but for our purposes it will be sufficient to assume that $\Phi_o = \zeta$. Later in this discussion we will describe the direct relationship that exists between Φ_o and the cell membrane surface charge density σ.

The inner and outer surface potentials, Φ_i and Φ_o, associated with fixed charges on the membrane surfaces and the transmembrane potential E_m are shown in Fig. 3a. In this figure the refer-

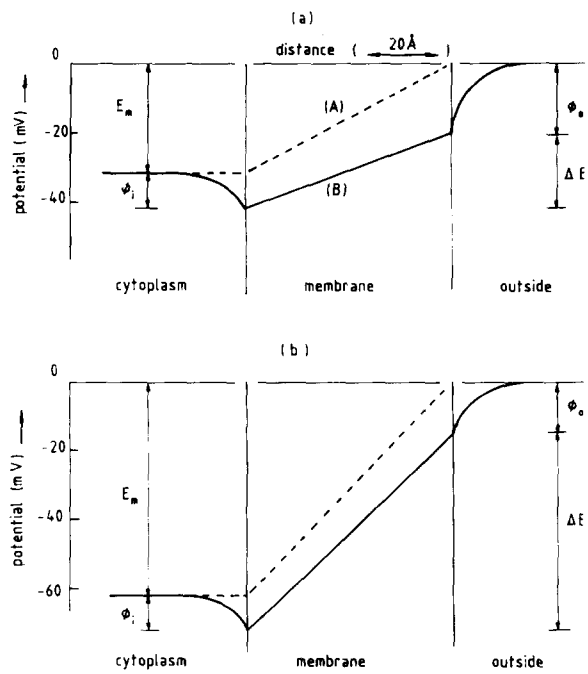

Fig. 3. Diagrams representing the potential profile across the plasma membrane of (a) transformed and (b) non-transformed 6m2 cells. The various symbols are explained in the text.

ence potential is taken to be that of the extracellular medium. The potential profile (A) can be derived from measurements using a microelectrode inserted into a cell, and is usually analyzed in terms of the ion diffusion model of Goldman-Hodgkin-Katz [37]. Profile (B) takes into account the electrical potentials associated with charges on the membrane surfaces, and the underlying polyelectrolyte theory that relates the surface charge density with the magnitude and shape of the surface potential has been described elsewhere [38,39]. The charge density on the internal membrane surface is taken as being lower than that of the external surface [39], so that Φ_o is shown larger in magnitude than Φ_i. From Fig. 3a it can be appreciated that the existence of fixed electrical charges on the membrane surfaces may have a significant influence on the magnitude of the actual potential difference ΔE_m acting across the membrane itself.

The increase shown in Figs. 1 and 2 of the zeta potential as the 6m2 cells changed from their transformed to non-transformed state is depicted in Fig. 3b as a decrease in the magnitude of Φ_o by 5 mV compared with its value of Fig. 3a. The scaling for E_m in Figs. 3(a) & (b) is based on the recent measurement [14] of -62 mV for NRK cells, as well as the 50% difference observed between non-transformed and transformed cells [11,14]. This variation in E_m on neoplastic transformation is also compatible with the 50% change in relative fluorescence shown in Fig. 2 for 6m2 cells.

In considering these fluorescent dye measurements it will be of value to discuss which features of the potential profile across the membrane have the greatest influence on the observed changes in fluorescence. The dye, diS-C_3-(5), used by Lai et al. [13] carries a net positive charge so that its distribution in the bulk phase on either side of the cell membrane is dependent on E_m [27]. The ratio N_i/N_o of the equilibrium concentrations of cationic dye molecules in the bulk phases inside and outside of the cell is given by the Boltzmann distribution

$$N_i/N_o = \exp(qE_m/kT) \tag{2}$$

In this equation k is the Boltzmann constant, q is the electronic charge ($-1.6 \cdot 10^{-19}$ C) and T is the absolute temperature. The concentration N_{is} of cationic dye located right at the inner membrane surface will be related to N_i by the equation

$$N_{is} = N_i \cdot \exp(q\Phi_i/kT) \tag{3}$$

where Φ_i is the electrostatic potential due to the presence of fixed electrical charges on the cell's inner membrane surface. It is assumed [27,28] that the decrease in fluorescence is largely proportional to the extent of dimerization of the dye molecules at the inner membrane surface. The extent of dimerization will be proportional to n_{is}^2, so that from Eqns. 2 and 3 the relative reduction in fluorescence will be proportional to the factor $N_o^2 \cdot \exp[2q(E_m + \Phi_i)/kT]$. This shows that the fluorescent dye technique closely monitors changes in the total potential ($E_m + \Phi_i$) at the inner membrane surface. A combination of the invasive microelectronic technique, which determines E_m alone, with the fluorescent dye measurements could therefore allow an estimate of the potential Φ_i to be obtained. This valuable aspect of the fluorescence technique in providing data for the internal membrane surface, being complimentary to that obtained by electrophoresis for the outer surface, does not appear to have been generally appreciated.

The results of Fig. 2 show that the 6m2 cells in the transformed state have a more negative Φ_o value and a smaller ΔE_m value (as shown in Fig. 3a) compared with the non-transformed state (Fig. 3b). It is of interest to relate the experimentally observed variation in the zeta potential of 6m2 cells as they undergo neoplastic transformation (Fig. 2) to the corresponding changes in their cell surface charge density. If the absolute value of Φ_o is less then 20 mV, then σ (μC/cm^2) is related [39,40] to the ionic concentration C_b (mol/liter) of the surrounding bulk aqueous mono-valent electrolyte by the expression

$$\sigma = -0.341 C_b^{1/2} \Phi_o q/kT^{1/2} \tag{4}$$

The presence of small concentrations of divalent ions in the phosphate-buffered saline used in this work can be shown to have negligible effect on the magnitude of σ derived from Eqn. 4. The total

ionic strength was calculated to be 180 mM and this was used as the value for C_b in Eqn. 4. Using the zeta potential values shown in Fig. 1 for Φ_o in Eqn. 4, then the NRK cells and non-transformed 6m2 cells at 39°C can be calculated to each have a net surface charge density of -1.42 $\mu C/cm^2$, whilst the transformed 6m2 cells at 33°C have a surface charge of -1.85 $\mu C/cm^2$. As the temperature of the 54-5A4 cells is decreased from 39°C to 33°C their surface charge changes from -1.78 to -1.72 $\mu C/cm^2$. The results of the RNAase-treatments summarized in Table II indicate that of the total increase in negative charge (0.43 $\mu C/cm^2$) that appears on the surface of the 6m2 cells as they change from their non-transformed to transformed state, around 72% (0.31 $\mu C/cm^2$) is associated with RNAase-sensitive entities. For a typical cell diameter of 15 μm, then assuming that these entities are RNA fragments this change in surface charge of 0.31 $\mu C/cm^2$ is equivalent to there having been $1.4 \cdot 10^7$ RNA nucleotides on the surface of each transformed 6m2 cell. For comparison, NRK cells typically contain 0.87 pg RNA [41], which corresponds to $1.8 \cdot 10^9$ RNA nucleotides per cell.

Conclusions

Electrophoresis measurements on the 6m2 cells have shown that the surface charge (-1.85 $\mu C/cm^2$) on those of transformed phenotype is more negative than that determined (-1.42 $\mu C/cm^2$) for their non-transformed counterparts. The irreversibly transformed 54-5A4 cells were also found to be more negatively charged than normal rat kidney cells. This finding that the cell surface charge becomes more negative on neoplastic transformation is in agreement with the results of Ambrose et al [15,16] obtained for a variety of non-transformed and homologous tumor cells. The effect of RNAase treatment indicates the interesting possibility that around 72% of the difference in surface charge between non-transformed and transformed 6m2 cells is associated with the presence of RNA. Although this finding may be surprising, we note that RNA has been reported [31,34,42,43] to contribute to the net surface charge of various ascites and sarcoma cells.

The fluorescence measurements [13] indicated that, when the temperature of the 6m2 cells was shifted from 33°C to 39°C, their transformation to the non-transformed phenotype was accompanied by a significant increase in the absolute magnitude of E_m. Our results show that together with the increase in E_m there is a decrease in the amount of cell surface charge. By contrast, when the temperatures of the NRK and 54-5A4 cells were changed from 33°C to 39°C, the fluorescence data [13] indicated that relatively small changes occurred in E_m, and our results show that the surface charge also remains relatively constant. The fluorescence data [13] for the 6m2 cells reinforces the concept that cancer (transformed) cells are more depolarized than their non-transformed counterparts. The few observed exceptions to this (see, for example, Refs. 1–3) should, however, not be overlooked.

At 33°C the 6m2 cells have been observed [44] to synthesize an 85 kDa polyprotein ($P85^{gag\text{-}mos}$). This protein is coded for by RNA from the ts110 sarcoma virus and disappears after the temperature is shifted from 33°C to 39°C. The disappearence of this protein and the morphological changes that accompany the shift of the 6m2 cells to 39°C take place on the same time scale as the changes in transmembrane and zeta potentials shown in Fig. 2. The appearance of RNA on the transformed 6m2 cells could be associated with the synthesis of this polyprotein.

The absolute magnitude of E_m for non-transformed cells has been observed to increase as the cell number density increases, whereas for cancer cells E_m is unrelated to cell density [3,14,20]. It is also of interest to note that NRK cells, infected with a temperature-sensitive mutant of avian sarcoma virus, exhibited [45] a rapid decrease in cell-to-cell junctional permeability on changing from their non-transformed to transformed phenotype, and E_m has been shown to modulate the cell-to-cell junctional conductance in cell pairs isolated from the Chironomus salivary gland [46]. Evidence has also been obtained supporting the concept that the E_m changes that accompany transformation of the 6m2 cells reflect a conformational and functional alteration in ($Na^+ + K^+$)-ATPase [47]. Intercellular communication, the transmembrane potential, cell surface charge and active ion transport may therefore all be interre-

lated and act as regulators of neoplastic transformation. Clearly, further studies are required before any real understanding of such possible interrelationships can be attained. More investigations are also needed of the possible influence of changes of cell morphology on electrophoresis measurements, and how factors such as changes in cell adhesion that accompany neoplastic transformation are related to changes in surface charge [48].

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