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Genes for Neuronal Properties Expressed in Neuroblastoma × L Cell Hybrids

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ABSTRACT **Neuroblastoma cells with electrically excitable membranes were fused with electrically passive L cells having a hitherto undescribed electrical marker. Hybrid cells, examined 10–40 generations after fusion, were found to be electrically excitable. The results show that at least a part of the genetic information for neuron differentiation can be functionally expressed in N × L hybrid cells. Evidence for the regulation of action potential components was also found.**

Clonal lines of mouse neuroblastoma cells thus far have been shown to possess ten properties characteristic of differentiated neurons including electrically excitable membranes and acetylcholine receptors (1–9). Some of the properties also are responsive to regulatory influences (4, 5, 9). When the number of neuronal functions expressed is considered, it is likely that the cells follow a genetic program for neuron differentiation. The neuroblastoma system thus may be useful in exploring steps in the maturation process as well as aspects of neural function.

The techniques of somatic cell hybridization have been used to probe the expression of the differentiated state with various cell types. Questions of dominance, complementation, and gene segregation can be studied (10–18,24,25). Also, the generality of control mechanisms can be tested by comparing intra- with interspecific hybrids.

In this report, somatic cell hybrids of mouse neuroblastoma and L cells are shown to possess the neuronal property of electrically excitable membranes.

MATERIALS AND METHODS

Cell lines

Mouse (C3H/AN) L cell mutant clones B82 and A9 were the gift of Dr. J. Littlefield (10/20/69). B82 lacks thymidine kinase (EC 2.7.1.21); A9 lacks hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) (15). We have detected no revertants and none have been reported in the literature. Mouse (A/J) neuroblastoma C-1300, clone N4, was derived by Dr. T. Amano from a single cell isolated from cells adapted to tissue culture.

Mutant production

Neuroblastoma N4, passage 36, was treated with 5×10^{-3} M ethyl methane sulfonate (Eastman Kodak Co.) for 2 hr to kill 60% of the cells, grown for five generations, and then exposed to 10^{-6} M 6-thioguanine (6-SGua) (Sigma Chemical Co.). Survivor frequency was approximately 2×10^{-5}. The survivors were pooled, grown up, and then exposed to 5×10^{-5} M 6-SGua. The frequency of resistant colonies was approximately 4×10^{-6}. Cells were pooled and then cloned in 10^{-4} M 6-SGua. One clone, N4TG1, selected for the present study, was maintained in 10^{-4} M 6-SGua, but was also shown to be resistant to 6-SGua after growth for several weeks in its absence. At the time of fusion, N4TG1 was approximately 80-100 generations removed from neuroblastoma N4. The reversion frequency was determined at the time of each fusion experiment by incubating 2×10^6 cells/60-mm plate and then subjecting the cells to the standard fusion procedure (see Table 1).

Media

Parental cells were cultured in medium D: Dulbecco's modification of Eagle's medium; 10% fetal calf serum, sodium penicillin G (50 units/ml), and streptomycin sulfate (10 μg/ml) in Falcon flasks or Petri dishes at 37°C in an atmosphere of 10% CO_2: 90% air, 100% humidity. Hybrid cells were grown in HAT medium: medium D, supplemented with 1×10^{-4} M hypoxanthine, 1×10^{-5} M aminopterin (a gift of Lederle Pharmaceutical Co.), and 1.6×10^{-5} M thymidine. Glycine (4×10^{-4} M) is present in medium D.

Cell fusion

An inoculum of Sendai virus, obtained from Dr. A. Rabson, was grown in embryonated chicken eggs, harvested, inactivated with β-propiolactone, and titered for hemagglutinating activity against guinea pig red blood cells (17, 18). Cell lines to be fused were mixed and immediately poured into a 60-mm Falcon Petri dish (2×10^6 total cells/5-ml medium D per dish). After 18-20 hr of incubation, cells were fused with 200-500 hemagglutinating units (HAU) of inactivated Sendai virus per dish according to the method of Ephrussi and Davidson (17). Plates were incubated for 15–20 days in HAT medium. Colonies containing approximately 1000 cells were counted, the medium was removed, and well-isolated large colonies were picked with the aid of disposable Pasteur pipets.

Electrical studies

The cells were maintained in medium D supplemented as follows: 1×10^{-4} M 6-SGua for N4TG1 and A9; 1×10^{-4} M BrdU (Sigma) for B82; HAT medium for hybrid cell lines. Cells from 50–100% confluent cultures were dissociated with 0.05% trypsin, centrifuged, resuspended, and then inoculated into 60-mm Falcon Petri dishes (1×10^5 cells/5 ml per dish)

TABLE 1. *Cell lines*

Cell lines*	Fusion ratio	Glucose phosphate isomerase phenotype† *a*	*h*	*b*	Base analog resistance (10^{-4} M)	Reversion frequency
Neuroblastoma, N4TG1		+	−	−	6-thioguanine	$\sim 1\text{–}5 \times 10^{-7}$
L-cell, A9		−	−	+	6-thioguanine	$< 5 \times 10^{-9}$
L-cell, B82		−	−	+	5-bromodeoxyuridine	$< 5 \times 10^{-9}$
Neuroblastoma × L-cell	(N4TG1 × B82)					
NL-1 hybrid	1:20	+	+	+		
NL-2 hybrid	1:200	+	+	+		
NL-3 hybrid	1:20	+	+	+		
NL-4 hybrid	1:20	+	+	+		
NL-5 hybrid	1:0.7	+	+	+		
NL-6 hybrid	1:0.7	+	+	+		
NL-7 hybrid*	1:200					
L-cell × L-cell	(A9 × B82)					
LL-1 hybrid*	1:20	−	−	+		
LL-2 hybrid*	1:20					

* Cell lines are clones except NL-7 (13 clones pooled); LL-1 (15 clones pooled); LL-2 (14 clones pooled). The probability of a revertant occurring in NL-7 is ~0.005. Designations in our laboratory for the cell lines are: N4TG1 (N16604B); NL-1 (23406B), NL-2 (23407C) NL-3 (23406C), NL-4 (23406D), NL-5 (22014A), NL-6 (22016A), NL-7 (23409).

† Starch gel electrophoresis of glucose phosphate isomerase. Bands *a*, *h*, and *b* migrated 1.2, 1.8, and 2.3 cm from the origin toward the cathode. Band nomenclature (19) is as follows: GPI-1A = *a* band alone; GPI-1B = *b* band alone; GPI-1AB = *a*, *h*, *b*, bands.

without 6-SGua or BrdU. After 24 hr, the medium was removed, plates were washed once with medium minus serum, and 5 ml of the following was added per plate: medium D minus serum for N4TG1, B82, A9; or HAT medium minus serum for hybrid cells.

Multiplication of neuroblastoma cells is dependent upon serum (4). Cells were incubated without serum to restrict cell division and shift the cells to a more differentiated state; i.e., an increase in specific activity of acetylcholinesterase and axon extension (4, 5). After 2–4 days incubation without serum, cells were used for electrical studies. In preliminary experiments neuroblastoma cells were found to be electrically active after incubation without serum.

The methods for studying cells with intracellular microelectrodes are described elsewhere (2,6). Use of an intercellular electrode in a bridge circuit allows measurement of cell membrane potentials and stimulation of the cell with intracellularly applied current. Transmembrane voltages and stimulating currents were digitized and stored with a Digital Equipment Corp. PDP-12 computer along with calculated values of the first derivative of membrane potential with respect to time. These parameters were then used to compute active and passive cell membrane properties.

Glucose phosphate isomerase phenotypes

Glucose phosphate isomerase (EC 5.3.1.9) phenotypes were determined by starch gel electrophoresis (19). Glucose-6-phosphate dehydrogenase (crystallized once) and fructose-6-phosphate (contaminated with less than 2% glucose-6-phosphate) were obtained from Sigma Chemical Co. Homogenates were prepared as previously described (5).

RESULTS

Formation of hybrid cells

In the presence of aminopterin, an inhibitor of nucleoside synthesis, cell growth depends upon the availability of preformed bases and the ability to synthesize enzymes required for base utilization. A 6-SGua resistant neuroblastoma mutant (N4TG1) unable to utilize hypoxanthine was obtained and Neuroblastoma × L cell hybrids containing genetic information from both parents within a common nucleus were selected by growth in HAT medium. The frequency of putative neuroblastoma × L cell (NL) hybrids per input neuroblastoma parental cell depended on the initial ratio of the parental cells; i.e., approximately 3×10^{-5} for 1:1 (B82: N4TG1); 5×10^{-4} for 20:1; 1.5×10^{-3} for 200:1.

Neuroblastoma and L cell lines are derived from A and C3H mouse strains, respectively, which express different glucose phosphate isomerase isozyme phenotypes at the *Gpi-1* locus (19). As shown in Table 1, each NL hybrid clone expresses the *Gpi-1* isozymes of both neuroblastoma and L cell parents and at least one additional isozyme as previously described in F_1 animals heterozygous for this locus (19). A mixture of nonhybrid N4TG1 and B82 cells, grown in the same vessel for 1 week, contained only the two parental bands (*a* and *b*), not the intermediate band (*h*). These results show that the clones are indeed hybrids of neuroblastoma and L cells. No revertants of parental L cell lines have been found. Hence, growth in HAT medium of fused L cells (B82 × A9) indicates that these cells are also hybrid (14,16).

Electrical properties

The electrophysiological properties of single cells were studied by inserting the tip of a microelectrode within a cell and measuring the voltage difference between this intracellular electrode and a second electrode immersed in the extracellular fluid. Pulses of current were then passed through the electrodes and the change in voltage across the cell membrane was measured as a function of time. These responses can be used to characterize both the active (excitable) and the passive properties of the cells (6).

Pulses of current which decrease the transmembrane voltage (depolarizing stimuli) provide a test for electrically active

TABLE 2. *Response of cells to electrical stimulation*

Cell lines	Post fusion Generations	Post fusion Days	Responses A^-B^- (P)	A^-B^+ (DR)	A^+B^- A^+B^+ (AR)	Total cells	C^-	C^+ (HA)	Total cells
						Number of cells			
Parents									
Neuroblastoma, N4TG1			52	8	8	68	36	0	36
L-cell, B82			20	0	0	20	2	8	10
L-cell, A9			16	0	0	16	0	4	4
NL hybrids									
NL-1	25–40	50–76	7	3	11	21	11	5	16
NL-2	25–35	50–76	7	13	3	23	16	5	21
NL-3	25	51	5	0	3	8	5	2	7
NL-4	25	50	9	1	0	10	2	1	3
NL-5	30	65	1	1	3	5	2	1	3
NL-6	30	65	1	3	5	9	4	2	6
NL-7	20	42	18	3	3	24	4	4	8
NL plate-1	10	20	12	1	15	28			
NL plate-2	10	26	1	4	6	11	4	1	5
NL plate-3	10	23	9	4	5	18	7	1	8
LL hybrids									
LL-1	25	30	11	0	0	11	2	9	11
LL-2	25	30	10	0	0	10	0	6	6
LL plate-1	10	15	10	0	0	10			0
				% of total		*Total cells*	*% of total*		*Total cells*
Totals									
Neuroblastoma			76	12	12	68	100	0	36
L-cells			100	0	0	36	14	86	14
NL hybrids	10		39	16	45	57	85	15	13
NL hybr ds	20–40		48	24	28	100	69	31	64
LL hybr'ds	10–25		100	0	0	31	12	88	17

Responses were scored as described in the results and Figs. 1 and 2. The abbreviations in parentheses are: P, passive; DR, delayed rectification; AR, active response; HA, hyperpolarizing activation. Hybrid cells assayed 10 generations after fusion were studied on the fusion plate. The number of colonies tested on each plate and the probability that a colony was a revertant were: NL plate-1, 3 colonies, 0.017; NL plate-2, 4 colonies, 0.012; NL plate-3, 2 colonies, 0.0008. The revertant probability was calculated by multiplying the reversion frequency of N4TG1 in HAT medium (obtained at the time of fusion) by the number of N4TG1 cells per fusion reaction, divided by the number of putative hybrid colonies found. Similar responses were obtained with different NL colonies on the same plate. Data for each plate are pooled. Each colony contained cells with A^-B^+ response; A^+ response was found in 8 of the 9 colonies. Some L cells and LL cells were tested with medium plus serum, but still were A^-B^-.

cell membranes. Each cell was tested for active responses at the resting membrane voltage (about 20–40 mV) and with the membrane voltage adjusted to a standard level (about 80 mV). This standard voltage was optimal for eliciting maximal responses and necessary for comparing cell types (2,6).

Three types of response to electrical depolarizing stimuli are shown in Fig. 1, *A–D*. The response from a cell that was not excitable, termed passive response (A^-B^-), is shown in Fig. 1*A*. A pulse of current elicited a smoothly rising uninflected change in membrane voltage. The *B* response (A^-B^+), termed delayed rectification, is scored when a negative inflection is present late in the response (Arrow *B* in Fig. 1*B*). Response *A* (A^+B^- or A^+B^+) is scored when a positive inflection (an increase in rate of voltage change) occurs early in the response (Arrow *A* in Fig. 1*C* and *D*). The two *A* responses (Fig. 1*C* versus 1*D*) differ quantitatively; the *A* response of Fig. 1*D* corresponds to an action potential, while that of Fig. 1*C* corresponds to a local response. The amount of *A* activity per cell varied over a wide range. Thus, quantitative as well as qualitative differences were observed.

A type of response not previously described was found with L cells but not with neuroblastoma cells (Fig. 2*A* and *B*). When a large (5–50 NA) 100-msec pulse of current, opposite in direction to that used in Fig. 1 *A–D*, was passed across the membrane a large (200–400 mV) increase in membrane potential resulted. After the current was turned off, the membrane potential returned to its initial value, and then underwent a second, prolonged (10–20 sec) increase, with subsequent return to the resting level (Fig. 2*A*). The voltage change was accompanied by a large decrease in cell resistance. We have termed this response the *C* response (hyperpolarization activation). The threshold for eliciting the *C* response was relatively sharp, and some increase in the threshold was seen after a *C* response; that is, cells were refractory.

The responses of neuroblastoma, L cell, and hybrid cell lines to electrical stimulation are shown in Table 2. The maximum responses of individual cells are shown.

Cells from the parent neuroblastoma clone were found that either exhibited no response (A^-B^-), the *B* response (A^-B^+), or the *A* response (A^+B^- or A^+B^+). The parent, therefore,

resembles the wild type neuroblastoma in that it expresses electrically active membranes. However, the incidence of passive cells (A^-B^-) was much greater than previously noted (6). None of the parental neuroblastoma cells gave a C response.

No L cell tested showed A or B responses; all were passive. However, nearly all the L cells exhibit the C response. Thus, A and B responses are neuroblastoma markers, whereas the C response is an L cell marker.

Six clonal lines of neuroblastoma × L cell hybrids and one uncloned NL hybrid line were tested 20–40 generations after fusion. Cells with excitable membranes were found with every NL hybrid line. With only one exception (NL-4), the incidence of expression of A or B responses found with NL hybrid lines equaled or exceeded that found with the neuroblastoma parent. With most NL hybrid lines passive cells, B, and A responding cells were found. The C response was also found in all N × L hybrid clones.

Nine colonies of putative NL hybrids were studied 10 generations after fusion directly on the fusion plate. The incidence of the B and A responses were again comparable to the neuroblastoma parent.

Thirty-one LL hybrid cells were tested 10–25 generations after fusion; the C response was found, but not A or B responses.

The overall incidence of expression of A and B responses was approximately twice as great in NL hybrids as in the neuroblastoma parent.

When a pulse of current is passed across the cell membrane so as to increase the potential difference across it, the voltage changes can be used to determine passive membrane properties such as resistance, time constant, and capacitance. The cell resistance is equal to the change in voltage divided by the amount of current (Fig. 3A); the membrane capacity is equal to the time constant (Fig. 3B) divided by the membrane resistance. A measure of the intensity of the A response is provided by the amount of current generated by a cell during the response, termed the action potential current (this is approximately equal to the product of the membrane capacity and the maximum rate of change of membrane voltage during the response).

The average action potential current of the NL hybrid cells scored with A responses is compared to that found with neuroblastoma cells (Table 3). Neuroblastoma cells with A responses had an average action potential current of 13 μA/cm^2; the most active cell was 25 μA/cm^2. In 3 of the 6 NL hybrid clones 20-40 generations after fusion, the maximum activity was higher (peak value, 85 μA/cm^2) than that found with the neuroblastoma parent, and the average action potential current was 22 μA/cm^2. Thus, the A response of hybrids equaled or exceeded that of parent neuroblastoma cells. Neuroblastoma cells displayed low specific resistivity and high specific capacitance compared to L cells and LL hybrids. High capacitance suggests either that the surface area of cells is greater than that estimated or that cells are coupled electrically (7).

Some correlation between electrical properties and the morphology of the cells in lines NL-1 and NL-2 was noted.

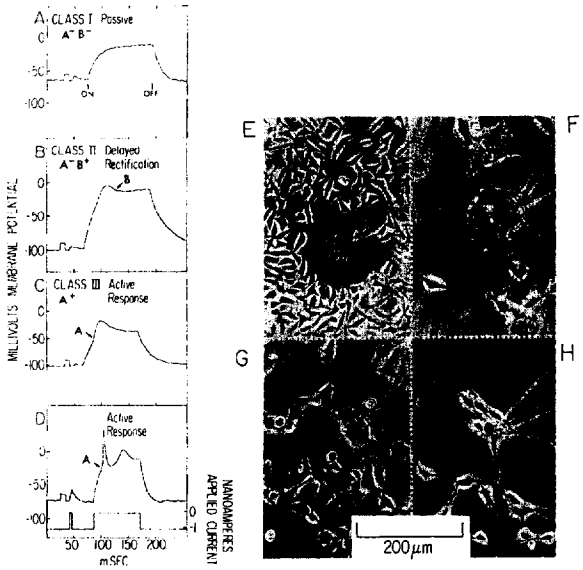
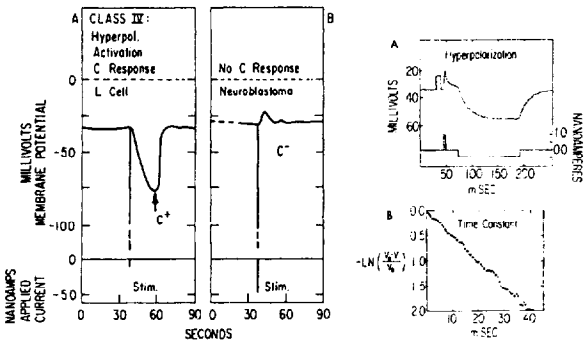


FIG. 1. Responses to electrical stimulation. A, B, C, and D are examples of the different categories of response to stimulating current, discussed in the text. The cell membrane potential is shown in each case as a function of time. A voltage calibration pulse and the response to a current calibration pulse are shown at the left of each trace, and the response to a long pulse of current occurs in the middle of each trace. Onset and cessation of current are indicated by *ON* and *OFF* in Fig. 1A, and the time course of the current is shown in the lower part of Fig. 1D. A) Passive response with no inflections (A^-B^-). B) Delayed rectification; a negative deflection occurs at the arrow, and in no other place, defining the B property (A^-B^+). C) and D) Active responses; a positive inflection occurs on the rising phase of the response, this defines the A property (A^+). Inflections may occur elsewhere. The response in Fig. 1C corresponds to a local or partial response; Fig. 1D represents an action potential. E, F, G, and H show cells under the conditions of electrical study, (medium minus serum for 3 days) with a microelectrode in place. Cell lines represented are: B82 A and E; NL-2 hybrid, B and F; N4TG1, C and G; *NL*-1 Hybrid, D and H.



(*Left*) FIG. 2. Hyperpolarization activation. Tracings of penwriter records show A) the presence of hyperpolarization activation or C response in an L cell, and B) the absence of a C response in a neuroblastoma cell.

(*Right*) FIG. 3. (A) Passive change in membrane voltage (*upper trace*) in response to hyperpolarizing current (*lower trace*). (B) Semilogarithmic plot obtained by evaluating the expression $-\ln (V_0 - V/V_0)$, where V_0 is the maximum voltage change developed in A, and V is the voltage occurring at the membrane. Time constant is equal to the time required for $(V_0 - V)/V_0$ to decrease by 1/e.

TABLE 3. *Membrane properties*

Cell line	Action potential current ($\mu A/cm^2$)	Specific resistivity (ohm cm^2)	Specific capacitance ($\mu F/cm^2$)	Membrane time constant (msec.)	C response (mV)	Maximum resting potential (mV)	Surface area (μm^2)	Total cells
Neuroblastoma, N4TG1	13	1600	7.3	7.3	0	24	15,400	14
L-cell, B82, A9 (average)	0	5100	2.9	8.8	30	47	12,480	15
NL-1 hybrid	19	3500	3.7	11.5	4	27	7,900	20
NL-2 hybrid	3.8	5500	3.5	15.5	3	34	13,900	23
LL-1,2 hybrids (average)	0	5200	4.1	11.2	20	42	12,770	21

Membrane properties of neuroblastoma and L-cell parents, 2 lines of NL hybrids, and 2 L-L hybrids. Action potential current refers to the average value for all cells in each line which showed the A^+ response. Surface area was determined by measuring cell body and process dimensions on photographs such as those shown in Fig. 1*E–H*. The average action potential current for all A^+ cells in NL hybrids 1–7 was 19 $\mu A/cm^2$.

Hybrid NL-1, which had extensive processes (Fig. 1*H*), also had low membrane resistivity and action potential current as high or higher than the neuroblastoma parent. Hybrid NL-2 consisted of large flat cells with no processes (Fig. 1 *E/B* & *F*). This line has high membrane resistivity as do the L cell lines. While the level of A^+ activity is low, the NL-2 line still had evidence of neuroblastoma function, as shown by the presence of the B^+ response (Table 2).

The *C* response was found with every NL hybrid clone, but less frequently than in L cells or LL hybrids. The average amplitude of the *C* response of NL hybrids was 10–20% that of L cells.

DISCUSSION

Neuroblastoma cells with electrically excitable membranes were fused with electrically passive L cells, and the resulting hybrid clones were selected by the procedure of Littlefield (15). Hybrid cells were examined 10–40 generations after fusion and found to be electrically excitable. The results show at least part of a genetic program for neuron differentiation can be functionally expressed in N × L hybrid cells. No evidence for a repressor terminating the neuron differentiation program was observed.

Since each assay for electrical activity is performed with a single cell, variation within a clone as well as variation between clones was studied. One disadvantage of this procedure is that cells are not randomly assayed since large cells can be studied more easily than small ones.

Two properties of electrically excitable membranes were assayed: a positive inflection on the rising phase and a negative inflection on the falling portion of the membrane-voltage curve, termed *A* and *B*, respectively. These correspond to the rising phase of the action potential and the descending phase of the action potential or delayed rectification. Three general categories of cells were found; cells without electrical activity (A^-B^-), cells exhibiting only *B* activity (A^-B^+), and cells with *A* responses (no attempt was made to distinguish between A^+B^- and A^+B^+ cells).

The three types of response were found with cells from almost every NL hybrid line, cloned and uncloned. A quantitative difference in the intensity of the *A* response also was found with cells from each of the 7 neuroblastoma clones that have been studied thus far, and with every N × L hybrid line studied. The simplest explanation of both quantitative and qualitative variation is that *A* and *B* activities are regulated and that B can be active independently of the *A* response.

Some chromosomes probably are lost by NL hybrids during the course of growth (12,13,16,23); however, no definitive evidence for gene segregation was found. Chromosomes are lost preferentially from the genome of the parent with the longer generation time (13). Neuroblastoma and L cell generation times are similar; thus, part of the genome of either parent may be lost from NL hybrids. Cell lines defective in neuronal properties would be useful both in elucidating steps that pertain to neuron maturation and in defining neural functions. The finding that NL hybrids often are more active electrically than the parental neuroblastoma line could be due to activation of L cell genes for action potential components, complementation, or other forms of regulation.

Transmission of an action potential by excitable cell membranes probably involves a series of reactions initiated sequentially by reactions of neighboring molecules. The available information on the nature of the action potential indicates that the *A* response results, at least in part, from Na^+ entry into the cell, and the *B* response from the exit of K^+ from the cell (20). The cations are transported with specificity, probably without a requirement for ATP; hence, transport by facilitated diffusion seems likely. Although the components required for the action potential have not been identified, it is possible that the *A* response is dependent upon a Na^+ entry permease which is converted reversibly from an inactive to an active form when cell membrane potential is decreased, and is inhibited by tetradotoxin, whereas the *B* response may require a K^+ exit permease inhibited by tetraethylammonium ions. It seems likely that other steps also are required. An alternate model for the production of the action potential has been proposed (22).

The relatively low frequency and amplitude of the *C* response found with NL hybrid cells may be due to repression of the L cell marker. Relatively few cells were found with C^+A^+, C^+B^+, or $C^+A^+B^+$ responses. The mechanism of the *C* response possibly involves an increase in membrane conductance to potassium.

The techniques of somatic cell hybridization applied to normal neuroblasts may well provide a relatively simple means of establishing clonal lines of cells derived from different types of neurons. The results obtained with neuroblastoma cells show that rapidly dividing cells still retain the ability to express neuronal function and that some neuronal genes

remain active in somatic cell hybrids. The possibility that neurons may be capable of initiating a program for neuron differentiation in recipient cells also deserves consideration.

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1. Augusti-Tocco, G., and G. Sato, *Proc. Nat. Acad. Sci. USA*, **64**, 311 (1969).
2. Nelson, P., R. W. Ruffner, and M. Nirenberg, *Proc. Nat. Acad. Sci. USA*, **64**, 1004 (1969).
3. Schubert, D., S. Humphreys, C. Baroni, and M. Cohn, *Proc. Nat. Acad. Sci. USA*, **64**, 316 (1969).
4. Seeds, N. W., A. G. Gilman, T. Amano, and M. Nirenberg, *Proc. Nat. Acad. Sci. USA*, **66**, 160 (1970).
5. Blume, A., F. Gilbert, S. Wilson, J. Farber, R. Rosenberg, and M. Nirenberg, *Proc. Nat. Acad. Sci. USA*, **67**, 786 (1970).
6. Nelson, P., J. Peacock, T. Amano, and J. Minna, *J. Cell. Physiol.* (in press).
7. Harris, A. J., and M. J. Dennis, *Science*, **167**, 1253 (1970).
8. Olmsted, J. B., K. Carlson, R. Klebe, F. Ruddle, and J. Rosenbaum, *Proc. Nat. Acad. Sci. USA*, **65**, 129 (1970).
9. Schubert, D., and F. Jacob, *Proc. Nat. Acad. Sci. USA*, **67**, 247 (1970).
10. Finch, B. W., and B. Ephrussi, *Proc. Nat. Acad. Sci. USA*, **57**, 615 (1967).
11. Kao, F. T., L. Chasin, and T. T. Puck, *Proc. Nat. Acad. Sci. USA*, **64**, 1284 (1969).
12. Weiss, M. C., and H. Green, *Proc. Nat. Acad. Sci. USA*, **58**, 1104 (1967).
13. Littlefield, J. W., *Science*, **145**, 709 (1964).
14. Davidson, R. L., B. Ephrussi, and K. Yamamoto, *Proc. Nat. Acad. Sci. USA*, **56**, 1437 (1966).
15. Littlefield, J. W., *Exp. Cell Res.*, **41**, 190 (1966).
16. Kao, F. T., and T. T. Puck, *Nature*, **228**, 329 (1970).
17. Davidson, R. L., *Exp. Cell Res.*, **55**, 424 (1969).
18. Harris, H., J. F. Watkins, C. E. Ford, and G. I. Schoefl, *J. Cell. Sci.*, **1**, 1 (1966).
19. De Lorenzo, R. J., and F. H. Ruddle, *Biochem. Genet.*, **3**, 151 (1969).
20. Hodgkin, A. L., and A. F. Huxley, *J. Physiol. (London)*, **117**, 500 (1952).
21. Cole, K. S., *Membranes, Ions, and Impulses*, U. of Cal. Press, Berkeley, Calif. (1968).
22. Tasaki, I., *Nerve Excitation*, C. Thomas, Springfield, Illinois (1968).
23. Engle, E., B. J. McGee, and H. Harris, *Nature*, **223**, 152 (1969).
24. DiZerega, G., and J. Morrow, *Exp. Neurology* **28**, 206 (1970).
25. Jacobson, C., *Exp. Cell Res.*, **53**, 316 (1968).