Mitogens and Oncogenes Can Block the Induction of Specific Voltage-Gated Ion Channels

JOHN M. CAFFREY, ARTHUR M. BROWN, MICHAEL D. SCHNEIDER*

The mechanisms underlying the ontogeny of voltage-gated ion channels in muscle are unknown. Whether expression of voltage-gated channels is dependent on mitogen withdrawal and growth arrest, as is generally true for the induction of muscle-specific gene products, was investigated in the BC_3H1 muscle cell line by patch-clamp techniques. Differentiated BC_3H1 myocytes expressed functional Ca^{2+} and Na^+ channels that corresponded to those found in T tubules of skeletal muscle. However, Ca^{2+} and Na^+ channels were first detected after about 5 days of mitogen withdrawal. In order to test whether cellular oncogenes, as surrogates for exogenous growth factors, could prevent the expression of ion channels whose induction was contingent on mitogen withdrawal, BC_3H1 cells were modified by stable transfection with oncogene expression vectors. Expression vectors containing v-*erb*B, or c-*myc* under the control of the SV40 promoter, delayed but did not prevent the appearance of functional Ca^{2+} and Na^+ channels. In contrast, transfection with a Val^{12} c-H-*ras* vector, or cotransfection of c-*myc* together with v-*erb*B, suppressed the formation of functional Ca^{2+} and Na^+ channels for ≧4 weeks. Potassium channels were affected neither by mitogenic medium nor by transfected oncogenes. Thus, the selective effects of certain oncogenes on ion channel induction corresponded to the suppressive effects of mitogenic medium.

MUSCLE DIFFERENTIATION IS ACcompanied by developmental changes in the expression of voltage-gated membrane ion channels (*1*), whose underlying cellular mechanisms are unknown. In contrast, the induction of genes that encode each subunit of the ligand-gated nicotinic acetylcholine receptor has been linked to cessation of cell division or to withdrawal of mitogenic growth factors (*2, 3*). It has been proposed that cellular oncogenes that have been implicated in mediating growth factor effects (*4*) function as negative regulators of myogenesis (*5*). Furthermore, certain variant muscle cell lines that cannot differentiate autonomously express either c-*myc* (*6*) or the epidermal growth factor (EGF) receptor encoded by c-*erb*B (*7*). We therefore examined the ontogeny of voltage-gated ion channels in BC_3H1 muscle cells, a clonal line that has been used extensively as a model of myogenic differentiation (*3, 5, 8, 9*). We wanted to determine whether expression of one or more voltage-gated channels in BC_3H1 myocytes would be contingent on mitogen withdrawal, or would be influenced by stable transfection (*10*) with cellular or viral oncogenes that mimic the effects of growth factors on muscle-specific gene induction (*9*). We report that expression of functional calcium and sodium channels was suppressed both during proliferative growth and by transfection with certain oncogene alleles. In contrast, expression of potassium channels was inhibited neither by mitogenic medium nor by the transfected oncogenes.

We examined Ca^{2+}, Na^+, and K^+ currents in whole cells or single channels by means of the gigaseal patch-clamp method (*11*). In BC_3H1 cells that had been induced to differentiate in 0.5% fetal bovine serum (FBS) for 5 days (*5, 9*) we identified (i) a rapidly activating, low-threshold Ca^{2+} current, (ii) a slowly activating, higher threshold Ca^{2+} current (Fig. 1), (iii) a delayed, outwardly rectifying K^+ current, and (iv) an inward Na^+ current that was half-maximally inhibited by tetrodotoxin at 10 μ*M*. Whole-cell recordings of the Ca^{2+}, Na^+, and K^+ currents, current-voltage (*I-V*) relations, and unitary channels are each shown in Fig. 1. The two Ca^{2+} currents resembled those reported for skeletal muscle (*12*) and differed from the Ca^{2+} currents reported for smooth muscle cells (*13*) (Fig. 1, A to C, and Table 1). The "slow" Ca^{2+} current of BC_3H1 cells activated with a time constant ≅20 to 100 msec, comparable to "slow" Ca^{2+} channels found in T tubules of skeletal muscle. The "fast" current inactivated very slowly, as observed in T tubules. Furthermore, the "fast" and "slow" components could be distinguished by the voltage dependence of inactivation (see the legend to Fig. 1B), conductance and mean open time of single channels (Fig. 1C), divalent cation selectivity, and pharmacology. Taken together, these data indicate that two independent classes of elementary Ca^{2+} channels coexist in differentiated BC_3H1 cells, whose biophysical properties correspond to those in T tubules of skeletal muscle. The kinetics and *I-V* relations of the K^+ current (Fig. 1, D to F) and Na^+ current (Fig. 1, G and H) also were similar to those reported for skeletal muscle (*14, 15*).

To determine whether changes in voltage-gated currents of BC_3H1 muscle cells could be induced by mitogen withdrawal, we compared whole-cell clamp recordings from exponentially growing cells with those from cells at 1 to 20 days in 0.5% FBS (Fig. 2). Ca^{2+} and Na^+ currents could not be detected in proliferating BC_3H1 myoblasts, at test potentials ranging from −20 to +60 mV: these currents first appeared after 4 to 5 days of mitogen withdrawal. Peak amplitudes for the three inward currents, relative to one another, neither varied appreciably from cell to cell nor varied during the observed time course of differentiation (*1*). Current densities were normalized to membrane area, which was estimated from both cell capacitance and cell geometry, and were found to increase through days 15 to 20 after mitogen withdrawal and then to plateau (Fig. 2B). We estimated channel number (*N*) using the relation $I = N \cdot i \cdot P_o$ where *I* denotes the amplitude of the whole-cell current, *i* is the mean single-channel amplitude, and P_o indicates the probability that a single channel is in an open state (*11*). For the "slow" Ca^{2+} current, at +10 mV in 50 m*M*

Table 1. Properties of Ca^{2+} channels in BC_3H1 myocytes, skeletal muscle (*12*), and smooth muscle (*13*). V_{th}, potential for threshold activation; V_p, potential at which inward current is maximal; and $t_{1/2}$, time to 50% activation or inactivation.

Samples	V_{th} (mV)	V_p (mV)	$t_{1/2}$ Activation (msec)	$t_{1/2}$ Inactivation (msec)
BC_3H1 myocytes				
Fast	−30 to −20	−10 to 0	2 to 10	500 to 5,000
Slow	−20 to 0	0 to +20	20 to 100	1,000 to 10,000
Skeletal muscle				
Fast	−70 to −60	−20 to −10	5 to 50	500 to 5,000
Slow	−50 to −40	−10 to 0	100 to 300	500 to 5,000
Smooth muscle				
Fast	−60 to −50	−20 to −10	1 to 10	10 to 50
Slow	−30 to −10	0 to +30	2 to 10	200 to 1,000

J. M. Caffrey and A. M. Brown, Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.
M. D. Schneider, Molecular Cardiology Unit, Departments of Medicine, Cell Biology, and Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.

*To whom correspondence should be addressed.

Ba^{2+}, $I \cong 400$ pA, $i = 0.65$ pA, and $P_o \cong 0.1$. Thus, we calculate that differentiated BC_3H1 myocytes may express $\sim 10^4$ functional "slow" Ca^{2+} channels per cell, with a doubling time of approximately 2 days during mitogen withdrawal. For the K^+ current, $I \cong 500$ pA, $i = 0.5$ pA, and $P_o \cong 0.5$, indicating that BC_3H1 cells express ~ 2000 K^+ channels per cell (Fig. 1, D to F). In contrast to our results for Ca^{2+} and Na^+ currents, the K^+ current was relatively invariant during differentiation (Fig. 2D).

To test if induction of Ca^{2+} channels can be inhibited via genes that encode proteins postulated to transduce or emulate mitogenic signals, we investigated clonal cell lines derived from BC_3H1 cells that had been stably transfected with activated alleles of c-*myc*, v-*erb*B, both genes, or c-H-*ras* (*9*) (Fig. 3). The c-*myc* expression vector, pSVc-*myc*-1, contained murine c-*myc* coding sequences under the transcriptional control of the SV40 promoter (*16*). The v-*erb*B vector, pAEV11-d1-3, consisting of the avian erythroblastosis viral genome deleted in the irrelevant *erbA* and *gag* sequences (*17*), corresponds to a truncated or ligand-independent EGF receptor. The vector, pEJ6.6, containing the Val^{12} allele of c-H-*ras*, encodes a *ras* protein that binds, but cannot hydrolyze guanosine triphosphate and is postulated to function constitutively in transduction of signals induced by growth factors (*16, 18*). Evidence that the transfected oncogenes are expressed in the resulting clonal cell lines is reported elsewhere (*9*). We found that either an autonomous c-*myc* gene or v-*erb*B delayed, but did not prevent, differentiation of BC_3H1 cells, indicated by >100-fold induction of muscle creatine kinase (*mck*) messenger RNA (mRNA) (*9*). In contrast, more than additive inhibition of differentiation resulted when c-*myc* was cotransfected with v-*erb*B. Moreover, the Val^{12} c-H-*ras* allele, when transfected alone, suppressed *mck* and α-actin mRNA induction (*9*). The expression vectors were not themselves mitogenic for BC_3H1 cells, and each of these stably transfected, clonal cell lines withdrew from the cell cycle within 48 hours of mitogen deprivation. Thus, the effects of transfected oncogenes on muscle-specific gene expression were independent of cell proliferation. In BC_3H1 cells that had been transfected with pSVc-*myc*-1 or pAEV11-d1-3, the initial expression of both Ca^{2+} channels was delayed by 1 to 2 days, when compared to control BC_3H1 myocytes. On the other hand, the Ca^{2+} current density ultimately was equivalent to that of the control cells (Fig. 2B). Neither the *I-V* relations nor the kinetics of the macroscopic Ca^{2+} currents were altered. These results agree with recent reports indicating that c-*myc* expression by itself does not suppress the differentiated phenotype in muscle cells (*9, 19*).

In contrast, cotransfection of pSVc-*myc*-1, together with pAEV11-d1-3, resulted in synergistic suppression of the two Ca^{2+} currents (Fig. 2). These results substantiate our previous observation that the concerted effect of the oncogenes c-*myc* and v-*erb*B on myogenesis was more than additive in these cells (*9, 11*). Consistent with its marked effects on muscle-specific gene induction (*9*), the Val^{12} allele of c-H-*ras* sufficed by itself to abolish expression of functional Ca^{2+} channels at up to 40 days in "differentiating" medium (Fig. 2). Maximal concentrations of isoproterenol ($10^{-6}M$) and BAY k 8644, a dihydropyridine agonist (DHP, $5 \times 10^{-6}M$) (*20*), did not elicit Ca^{2+} currents either in *ras*-transfectants or in proliferating cells. Since currents of ≧10 pA could be resolved, the number of Ca^{2+}

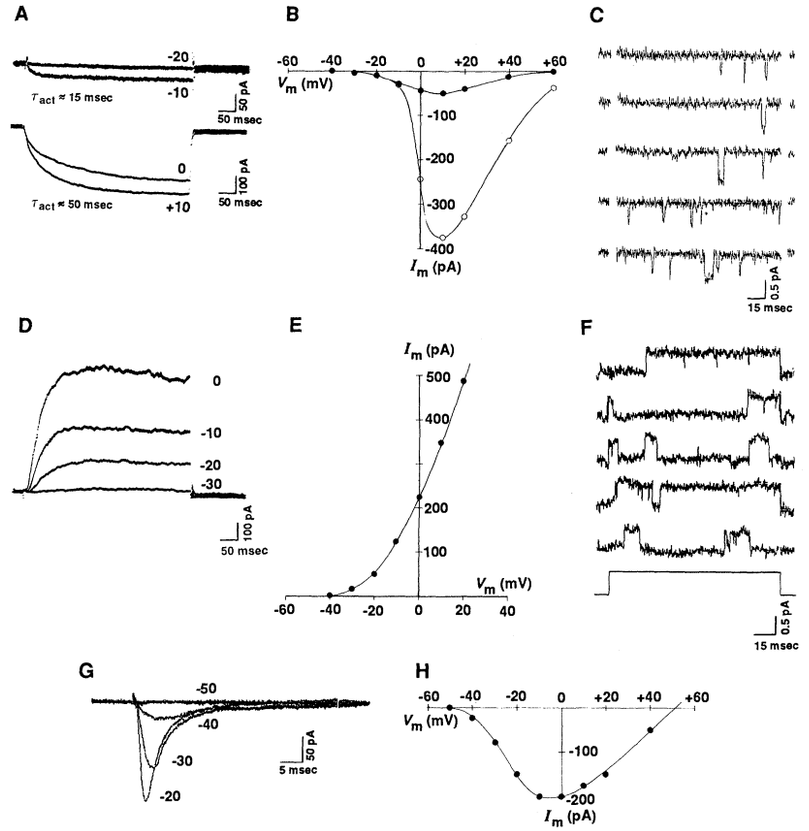

Fig. 1. Voltage-activated Ca^{2+}, K^+, and Na^+ currents in differentiated BC_3H1 myocytes. (**A**) "Fast," low-threshold (upper pair) and "slow," higher threshold (lower pair) inward Ba^{2+} currents were elicited by 450-msec voltage steps from −80 mV (V_h) to the test potentials (V_t) shown. The external solution was without Na^+ and contained: 20 m*M* barium glutamate, 110 m*M* *N*-methyl-D-glucamine, 5 m*M* glucose, and 10 m*M* Hepes (*p*H 7.3); cells were internally dialyzed with 130 m*M* cesium aspartate, 10 m*M* CsF, 5 m*M* EGTA, and 10 m*M* Hepes (*p*H 7.3). (**B**) Current (I_m) is plotted as a function of voltage of the membrane (V_m) for peak inward Ba^{2+} current elicited by voltage steps from −80 mV to the potentials shown (open symbols). In order to isolate the "fast" Ba^{2+} current, "slow" Ba^{2+} currents were inactivated with a holding potential of −50 mV (closed symbols) (*11*). (**C**) Single Ba^{2+} channels were measured in a differentiated BC_3H1 myocyte (cell-attached patch). Pipette: 50 m*M* barium glutamate, 50 m*M* tetraethylammonium glutamate, 15 m*M* glucose, and 10 m*M* Hepes (*p*H 7.3); bath: 50 m*M* potassium glutamate, 60 m*M* sodium glutamate, 5 m*M* glucose, and 10 m*M* Hepes (*p*H 7.3). Currents were elicited by 150-msec pulses from −100 to −10 mV at 5-second intervals, filtered at 1 kHz, and sampled at 100 μsec per point. (**D**) Delayed, outwardly rectifying K^+ currents were measured. $V_h = -80$ mV; $V_t = -30, -20, -10$, or 0 mV; the pulse duration was 450 msec. External: 5 m*M* $CaCl_2$, 130 m*M* NaCl, 5 m*M* KCl, 5 m*M* glucose, 10 m*M* Hepes (*p*H 7.3), and 5 m*M* Ca^{2+} Tyrode's solution; internal: 130 m*M* potassium aspartate, 10 m*M* KF, 5 m*M* EGTA, and 10 m*M* Hepes (*p*H 7.3). (**E**) Current (I_m) is plotted as a function of voltage of the membrane (V_m) for K^+ current 450 msec after onset of a voltage step from −80 mV to each potential. (**F**) Unitary K^+ channels were measured in a proliferative, undifferentiated BC_3H1 cell (cell-attached patch). $V_h = -80$ mV; $V_t = -20$ mV. Pulse duration was 150 msec. Pipette and bath: 5 m*M* Ca^{2+} Tyrode's solution. (**G**) Na^+ currents are depicted evoked by 45-msec voltage steps from −90 mV to the potentials shown. External: 5 m*M* Ca^{2+} Tyrode's solution; internal: 130 m*M* cesium aspartate, 10 m*M* CsF, 5 m*M* EGTA, and 10 m*M* Hepes (*p*H 7.3). (**H**) Current (I_m) as a function of voltage of the membrane (V_m) is plotted for peak inward Na^+ current elicited as in (G).

channels that were activated by DHP thus was decreased at least 50-fold by either FBS or transfection with the Val^{12} c-H-*ras* allele. Corresponding results were obtained for Na^+ currents (Fig. 2C), whereas, by contrast, the K^+ currents were not altered by any of the transfected genes (Fig. 2D).

It will be interesting to test the prediction that mitogenic stimulation and the transfected *ras* gene suppressed Ca^{2+} currents by channel biosynthesis, or because channels, though present, were rendered functionally inactive. It remains to be established whether an activated *ras* protein that can serve as a surrogate for exogenous growth factors (i) has a primary effect on induction of these three voltage-gated channels, or (ii) is coupled to channel function as a negative modulator (*21*), or (iii) acts principally through up-regulation of secondary autocrine factors. Our investigation does not resolve the issue of whether the effects of "activated" oncogene alleles, which have been altered by mutation or by transcriptional control, may be extrapolated to imply similar involvement of the respective endogenous genes. As illustrated by contrasting results in rat pheochromocytoma cells, biological responses to *ras*-dependent signals may reflect inherent properties of a particular cell lineage rather than properties intrinsic to the *ras* gene product (*22*).

Expression of K^+ channels in BC_3H1 cells was refractory to both the state of cell proliferation and to transfection with genes that suppress myogenic development. This "constitutive" expression of these K^+ channels agrees with previous findings of K^+ channel expression in immature or undifferentiated cells (*1, 23*) and contrasts with the regulated induction of functional Ca^{2+} and Na^+ channels.

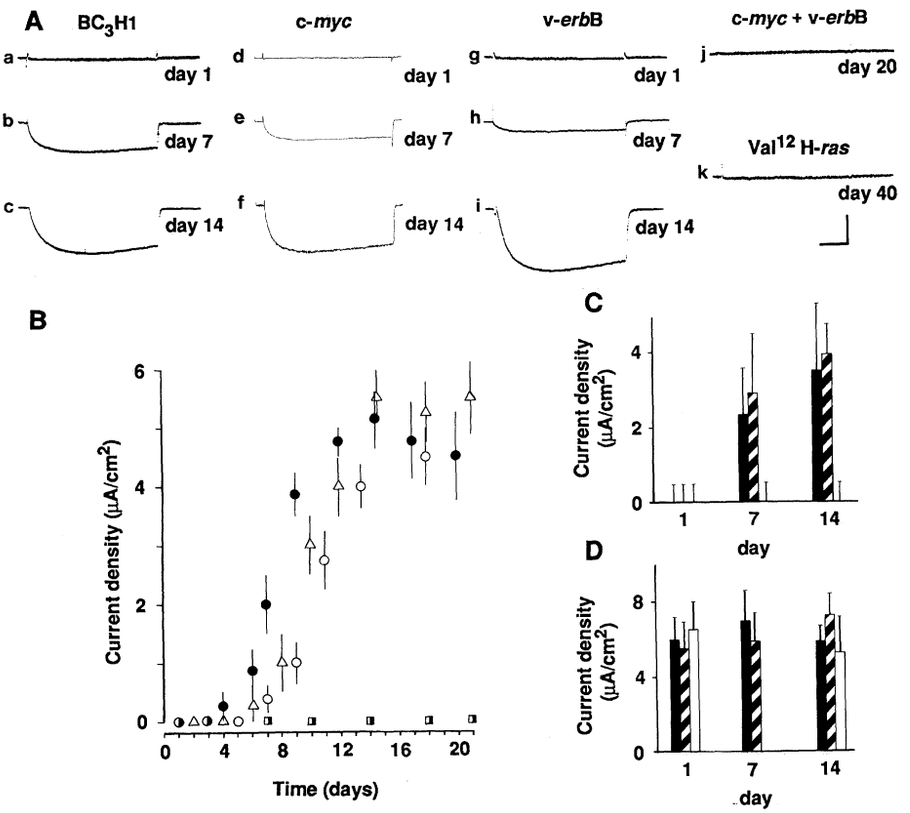

Fig. 2. (**A**) Induction of Ca^{2+} currents in BC_3H1 muscle cells after mitogen withdrawal and inhibition after transfection with genes that suppress myogenesis. Individual traces are shown for (traces a to c) BC_3H1 muscle cells or cells transfected with (traces d to f) c-*myc*, (traces g to i) v-*erb*B, (trace j) c-*myc* + v-*erb*B, or (trace k) Val^{12} H-*ras*. Cells were cultured in 0.5% FBS for the durations shown in days to the right of each tracing. Currents were elicited by voltage steps from −80 mV to a test potential of +10 mV (conditions as for Fig. 1A). Under these conditions, both the "fast" and "slow" components of the Ca^{2+} current are maximally activated in differentiated BC_3H1 myocytes (see Fig. 1B). Horizontal calibration: 50 msec; vertical: (day 1) 50 pA or (day 7 to day 40) 200 pA. (**B**) Ca^{2+} current density as a function of duration of serum withdrawal for BC_3H1 cells and transfectants. Values are means ± SD of peak Ba^{2+} current elicited as above. Cell surface area was calculated from each cell's input capacitance for 1 μF/cm² (*11*). Input capacitance for each cell line (~ 30 pF at day 1) was twice as much by day 14 for each transfectant except v-*erb*B, which was five times as much as on day 1. Results shown represent six independent experiments (three to eight observations per cell line for each time indicated after serum withdrawal). BC_3H1 (●), v-*erb*B (pAEV11-d1-3) (△), c-*myc* (pSVc-*myc*-1) (○), v-*erb*B + c-*myc* (□), Val^{12} c-H-*ras* (pEJ6.6) (■). (**C**) Na^+ current inactivation is dependent on mitogen withdrawal and is suppressed by an activated *ras* gene. Cells were shifted to 0.5% FBS for the duration shown in days under each set of bars. Na^+ currents evoked by pulses from −90 to −20 mV were normalized to membrane areas as for (B). BC_3H1 (solid); v-*erb*B (striped); Val^{12} c-H-*ras* (third or open). (**D**) Expression of the delayed rectifier K^+ current does not vary with the state of cell proliferation and is refractory to the transfected genes. Current densities were calculated as above, after mitogen withdrawal for the times shown. Bars as for (C).

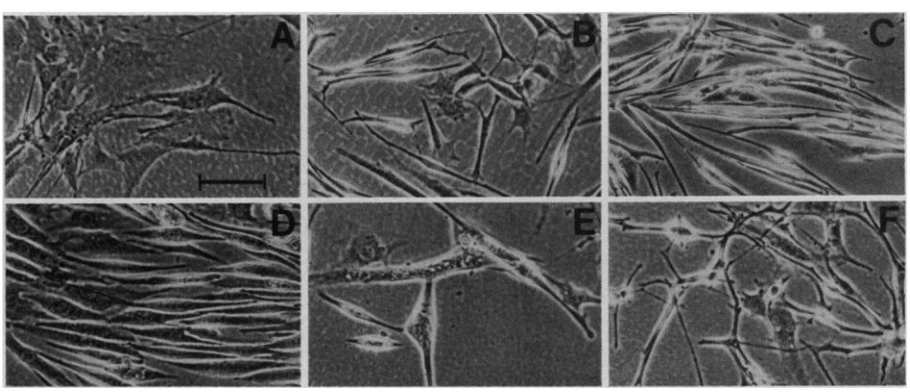

Fig. 3. Morphology of stably transfected BC_3H1 myocytes. Phase-contrast microscopy is shown for (**A**) proliferative BC_3H1 cells and (**B**) differentiated BC_3H1 cells, or after transfection with (**C**) c-*myc* (pSVc-*myc*-1); (**D**) v-*erb*B (pAEV11-d1-3); (**E**) c-*myc* + v-*erb*B; (**F**) Val^{12} c-H-*ras* (pEJ6.6). Panels B through F show quiescent cultures at 10 days of mitogen withdrawal. Bar = 100 μm. To introduce exogenous DNA into BC_3H1 muscle cells, we used transfection by the calcium phosphate method (*10*). Each reaction mixture for 10^6 cells per 100-mm dish comprised 1 μg of test DNA, 30 μg of carrier DNA, and 0.1 μg of pSV2*neo* DNA, conferring aminoglycoside resistance as a dominant selectable marker (*26*). Stable clones were isolated 18 to 21 days after transfection with the neomycin analog G418 at 400 μg/ml and were propagated in the selective medium. Measurements of oncogene incorporation and expression in the transfected myocytes are reported elsewhere (*9*).

In agreement with our results shown here for voltage-gated ion channels analyzed by patch-clamp techniques, transfection of BC_3H1 cells with the Val^{12} *ras* gene (but not the c-*myc* vector) was sufficient to prevent the induction of muscle differentiation products measured at the level of mRNA abundance. It is possible that the coordinate regulation of multiple channels, whose induction is contingent on mitogen withdrawal and is prevented by a single missense mutation, is interpreted most simply in the context of models for the control of muscle-specific gene induction via the recognition of shared, *cis*-acting DNA sequences (*24*). Since the regulatory events underlying the expression of voltage-gated channels during development are likely to be complex, it should prove advantageous to exploit a clonal system such as the present one, in which genetic manipulations produce clear-cut phenotypic effects (*25*).

REFERENCES AND NOTES

1. Y. Kidokoro, in *Excitable Cells in Tissue Culture,* P. Nelson and M. Lieberman, Eds. (Plenum, New York, 1981), pp. 319–339; D. Schubert, *Developmental Biology of Cultured Nerve, Muscle, and Glia* (Wiley, New York, 1984); A. Schmid, J.-F. Renaud, M. Fosset, J.-P. Meaux, M. Lazdunski, *J. Biol. Chem.* **259**, 11366 (1984); R. E. Weiss and R. Horn, *Science* **233**, 361 (1986).
2. J. P. Merlie, R. Sebbane, S. Gardner, E. Olson, J. Lindstrom, *Cold Spring Harbor Symp. Quant. Biol.* **48**, 135 (1983). For studies of genes encoding sarcomeric and cytosolic proteins: B. Nadal-Ginard, *Cell* **15**, 855 (1978); M. Shani *et al.*, *Dev. Biol.* **86**, 483 (1981); M. Caravatti *et al.*, *J. Mol. Biol.* **260**, 59 (1982); K. E. M. Hastings and C. P. Emerson, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1553 (1982); H. T. Nguyen, R. M. Medford, B. Nadal-Ginard, *Cell* **34**, 281 (1983); M. D. Schneider, P. A. Payne, H. Ueno, M. B. Perryman, R. Roberts, *Mol. Cell. Biol.* **6**, 4140 (1986).
3. L. Yu, R. J. LaPolla, N. Davidson, *Nucleic Acids Res.* **14**, 3539 (1986).
4. J. M. Bishop, *Cell* **42**, 23 (1985); H. E. Varmus, *Annu. Rev. Genet.* **28**, 553 (1984).
5. G. Spizz, D. Roman, A. Strauss, E. N. Olson, *J. Biol. Chem.* **261**, 9483 (1986); E. N. Olson, E. Sternberg, J. S. Hu, G. Spizz, C. Wilcox, *J. Cell Biol.* **103**, 1799 (1986).
6. T. Sejersen *et al.*, *J. Cell Physiol.* **125**, 465 (1985).
7. R. W. Lim and S. D. Hauschka, *Cold Spring Harbor Conf. Cell Proliferation* **9**, 877 (1982).
8. D. Schubert, A. J. Harris, C. E. Devine, S. Heinemann, *J. Cell Biol.* **61**, 398 (1974); E. N. Olson, K. C. Caldwell, J. I. Gordon, L. Glaser, *J. Biol. Chem.* **158**, 2644 (1983).
9. M. D. Schneider *et al.*, *Mol. Cell. Biol.*, in press; P. A. Payne *et al.*, *J. Cell Biol.* **103**, 30a (1986).
10. M. Wigler *et al.*, *Cell* **11**, 223 (1977); C. M. Corsaro and M. Pearson, *Somatic Cell Genet.* **7**, 603 (1981).
11. B. Sakmann and E. Neher, Eds. *Single Channel Recording* (Plenum, New York, 1983).
12. W. Almers and E. W. McCleskey, *J. Physiol. (London)* **353**, 585 (1984); ———, P. T. Palade, in *Calcium in Biological Systems,* R. P. Rubin, G. B. Weiss, J. W. Putney, Eds. (Plenum, New York, 1985), pp. 321–330; H. Affolter and R. Coronado, *Biophys. J.* **48**, 341 (1985); G. Cota and E. Stefani, *J. Physiol. (London)* **370**, 151 (1986).
13. J. M. Caffrey, I. R. Josephson, A. M. Brown, *Biophys. J.* **49**, 1237 (1986); B. P. Bean, M. Sturek, A. Puga, K. Hermsmeyer, *Circ. Res.* **59**, 229 (1986).
14. N. B. Standen, P. R. Stanfield, T. A. Ward, *J. Physiol. (London)* **369**, 339 (1985).
15. T. Gonoi *et al.*, *J. Neurosci.* **51**, 2559 (1985).
16. H. Land *et al.*, *Nature (London)* **304**, 596 (1983).
17. L. Sealy, G. Moscovici, C. Moscovici, J. M. Bishop, *Virology* **130**, 179 (1983); T. Gilmore, J. E. DeClue, G. S. Martin, *Cell* **40**, 609 (1985).
18. C. Shih and R. A. Weinberg, *Cell* **29**, 161 (1981); J. P. McGrath, D. J. Capon, D. V. Goeddel, A. D. Levinson, *Nature (London)* **310**, 644 (1984); R. W. Sweet *et al.*, *ibid.* **311**, 273 (1984).
19. T. Endo and B. Nadal-Ginard, *Mol. Cell. Biol.* **6**, 1412 (1986); G. Falcone, F. Tato, S. Alema, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 426 (1985); J. A. Coppola and M. D. Cole, *Nature (London)* **320**, 760 (1986).
20. A. M. Brown, D. L. Kunze, A. Yatani, *Nature (London)* **311**, 570 (1984); P. Hess, J. B. Lansman, R. W. Tsien, *ibid.*, p. 538.
21. G. G. Holz, S. G. Rane, K. Dunlap, *ibid.* **319**, 670 (1986).
22. M. Noda *et al.*, *ibid.* **318**, 73 (1985); D. Bar-Sagi and J. R. Feramisco, *Cell* **42**, 841 (1986).
23. L. Schlichter, N. Sidell, S. Hagiwara, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5625 (1986).
24. C. Emerson, D. Fischman, B. Nadal-Ginard, M. A. Q. Siddiqui, *Molecular Biology of Muscle Development* (Liss, New York, 1986).
25. M. Nirenberg *et al.*, *Science* **222**, 794 (1983).
26. E. M. Southern and P. Berg, *J. Mol. Appl. Gen.* **1**, 327 (1982).

27. We thank J. M. Bishop and R. A. Weinberg for the oncogene expression vectors, E. N. Olson for BC_3H1 muscle cells and his review of the manuscript, and K. Angelides, S. L. Mulvagh, and M. B. Perryman for suggestions. M.D.S. is grateful to R. Roberts and R. S. Adelstein for generous support and encouragement and to M. Nirenberg and H. Lecar for many helpful discussions. This investigation was supported by the following: grant to M.D.S. from the American Heart Association, Texas affiliate, 85G-223, and PHS grant from NIH, RR-05425; grants to A.M.B. from NIH, HL37044 and HL36475; and National Research Service Award to J.M.C., AR07546. M.D.S. and A.M.B. are investigators of the American Heart Association–Bugher Foundation Center for Molecular Biology of the Cardiovascular System.

3 November 1986; accepted 2 February 1987

Clustering of Genes Dispensable for Growth in Culture in the S Component of the HSV-1 Genome

RICHARD LONGNECKER AND BERNARD ROIZMAN*

The herpes simplex virus 1 genome consists of one long and one short stretch of unique sequences flanked by inverted repeat sequences. The nucleotide sequence and RNA map predict 12 open reading frames designated as US1 through US12 within the short stretch of unique sequences. This paper reports the construction of virus mutants from which US2, US3, or US4 had been deleted that are capable of growth in cell culture. One of the three deleted genes, US4, specifies the viral envelope glycoprotein G. Mutants with deletions in US1, US8, US9, US10, US11, and US12 have been previously reported. The nine genes deleted from this region form two clusters, US1 through US4 and US8 through US12, and encode at least two and possibly more structural proteins. The presence of so many genes dispensable for growth in cell culture suggests several hypotheses regarding their function and evolution.

THE 150-KILOBASE PAIR (KBP) HERpes simplex virus 1 (HSV-1) genome consists of two covalently linked components (L and S). Each component consists of unique sequences (U_l or U_s) flanked by inverted repeats (*1–3*). The inverted repeats of the L component, designated as *ab* and *b'a'* (*4*), each contain in their entirety two genes (α0 and γ34.5) (*5–7*); those of the S component, designated as *a'c'* and *ca*, each contain a copy of the α4 gene (5, 6) and an origin of viral DNA synthesis (*8–10*). The nucleotide sequence of the U_s sequence predicts the presence of 12 open reading frames designated as US1 to US12 (*11*). The products of several of these open reading frames have been identified as those of the α proteins α22 (US1) and α47 (US12) and of the glycoproteins G (US4), D (US6), and E (US8). Other studies have shown that virus mutants with deletions in the α22 gene or from which the entire domain of genes specifying gE (US8), US9, US10, US11, and US12 had been deleted are viable and multiply in cells in culture (*12, 13*). In this paper we report the deletion of DNA sequences encoding the open reading frames US2, US3, and US4. These genes are contiguous, mapping within the Bam HI N and Bam HI J fragments of HSV-1 strain F [HSV-1(F)] viral DNA (Fig. 1). On the basis of analyses of the nucleotide sequences, it was suggested that US2 specifies a membrane-translated protein (*11*) because of the predicted hydrophobic NH_2-terminal region, which could be a signal for membrane-bound translation. The predicted amino acid sequence for US3 is homologous to members of a protein kinase family in eukaryotes and retroviruses (*11, 14*). US4 encodes glycoprotein gG (*15, 16*).

The procedures used for construction of the deletion mutants were similar to those described previously (*12, 17*). Specifically, a chimeric thymidine kinase gene consisting of the promoter-regulatory domain of the α27 gene fused to the transcribed domain of

The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 East 58 Street, Chicago, IL 60637.

*To whom correspondence should be addressed.