**References and Notes**

1. C. B. Pert and S. H. Snyder, *Science* **179**, 1011 (1973).
2. C. Li, S. Lemaire, D. Yamashiro, B. Doneen, *Biochem. Biophys. Res. Commun.* **71**, 19 (1976).
3. J. Gautray, A. Jolivet, J. Vielh, R. Guillemin, *Am. J. Obst. Gynecol.* **129**, 211 (1977).
4. Y. Nakai, K. Nakao, S. Ohi, H. Imura, *Life Sci.* **23**, 2013 (1978).
5. E. Odagiri, B. Sherrell, C. Mount, W. Nicholson, D. Orth, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2027 (1979).
6. R. Simantov, S. Childers, S. Snyder, *Brain Res.* **135**, 358 (1977).
7. J. Loeber, J. Verhoef, J. Burbach, A. Witter, *Biochem. Biophys. Res. Commun.* **86**, 1288 (1979).
8. S. Nakanishi, H. Inoue, T. Kita, M. Nakamura, A. Chang, S. Cohen, S. Numa, *Nature (London)* **278**, 423 (1979).


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# Antiallergic Drug Cromolyn May Inhibit Histamine Secretion by Regulating Phosphorylation of a Mast Cell Protein


Abstract. *Cromolyn inhibited histamine release from mast cells that was induced by a classic secretagogue and correspondingly increased incorporation of radioactive phosphate into a 78,000-dalton protein. These effects on histamine secretion and on protein phosphorylation were rapid in onset and both showed tachyphylaxis. Cromolyn may therefore act by altering the phosphorylation of a protein involved in the regulation of secretion.*


Mast cells have been increasingly used as a model system for analysis of the cellular mechanisms involved in secretion (*1*) because they secrete by exocytosis, a mechanism common to many secretory cells (*2*), and, like most secretory cells, seemingly use calcium as a mediator in stimulus-secretion coupling (*3*). In addition, secretion by mast cells is of clinical interest because of its involvement in allergic diseases (*4*, *5*). In recent years it has been discovered that mast cell secretion can be inhibited by cromolyn sodium (cromoglycate) (*6*); this has led to a major advance in the prophylactic treatment of asthma (*7*).

Interest in discovering how cromolyn acts is intense, but little is known. It is evident that the action of cromolyn is not restricted to antigen-evoked secretion, for the drug can also potently inhibit, for example, mast cell secretion induced by the polyamine 48/80, the classic mast cell secretagogue (*8*). But further clues are sparse. Some authors (*6*) have speculated that cromolyn may be a "membrane stabilizer" and others (*9*) have suggested that it may block calcium entry. Here we present evidence that cromolyn may act to inhibit histamine release from mast cells by regulating phosphorylation of a specific mast cell protein. The experiments leading to this conclusion were prompted by the recent observation (*10*) that exposure of mast cells to 48/80 or the calcium ionophore A23187 alters the phosphorylation of several protein bands as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Since phosphorylation of three of these protein bands showed calcium dependence and occurred rapidly (while the secretory response was still developing), we suspected that these phosphorylation reactions might be involved in the induction of exocytosis and offer a clue to the still enigmatic role of calcium in stimulus-secretion coupling. In addition, the same experiments showed that a fourth protein band (78,000 daltons) incorporated radioactive phosphate later in the course of the response to 48/80, when secretion had largely run its course and the cells were presumably recovering. Here we report that exposure to cromolyn, whose effect is not to induce secretion but to inhibit it, results in selective phosphorylation of the same 78,000-dalton protein band. Together, these results suggest a possible association between phosphorylation of this particular protein band and the cellular mechanisms for physiological termination and pharmacological block of the secretory response.

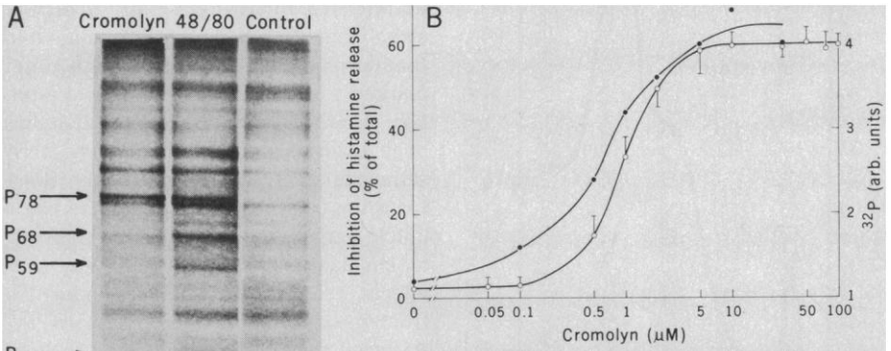


Fig. 1. (A) Autoradiograph showing the effect of cromolyn and of 48/80 on radioactive phosphate incorporation into proteins of intact mast cells. Mast cells (90 percent purity, $1.5 \times 10^6$ cells per milliliter) were first incubated in Hepes-buffered Locke's solution (*11*) with 0.75 mCi of carrier-free $^{32}$P-labeled inorganic phosphate (9120 Ci/mmole; New England Nuclear) per milliliter for 1 hour at 37°C, and were then incubated (0.2 ml per tube) without any drug or with cromolyn (10 $\mu M$) or 48/80 (1 $\mu$g/ml) for 1 minute at 37°C as described in (*10*). The cells were then dissolved in a solution containing 10 percent (weight to volume) sodium dodecyl sulfate (*10*), placed in boiling water for 2 minutes, and analyzed for radioactive phosphate incorporation by electrophoresis on 10 percent polyacrylamide gels and by autoradiography (*19*). (B) Effect of cromolyn in various concentrations on the incorporation of radioactive phosphate into the 78,000-dalton protein band and on histamine secretion from rat peritoneal mast cells. Incorporation of radioactive phosphate (•) was determined by scanning autoradiographs of the type shown in (A) with a Canalco G-II microdensitometer. These results were obtained in the absence of 48/80. Similar dose-response curves were obtained in two other experiments: in both instances there was increasing phosphorylation of the 78,000-dalton protein band over the same range of cromolyn concentrations. To measure mast cell secretion (○), approximately $10^5$ cells per milliliter were incubated simultaneously with 48/80 (1 $\mu$g/ml) and cromolyn (in the concentrations indicated) for 5 minutes at 37°C (*20*), and the histamine released was assayed fluorometrically in duplicate portions (*21*). Results represent mean ± standard error for five experiments.

Rat peritoneal mast cells were purified (90 percent) and incubated as described in (*11*). As a stimulus for histamine secretion, we used 48/80, which seemingly acts via receptors at the cell surface (*12*) to initiate exocytosis (*13*)—mainly by mobilizing cellular calcium (*14*). Mast cells which had been incubated for 1 hour with $^{32}$P-labeled inorganic phosphate to label intracellular adenosine triphosphate (*10*), and then incubated with cromolyn for 60 seconds at 37°C, showed increased incorporation of radioactive phosphate into a single protein band with an apparent molecular weight of 78,000 (Fig. 1A, compare cromolyn and control lanes). This molecular weight is similar to that of the protein phosphorylated relatively late in the



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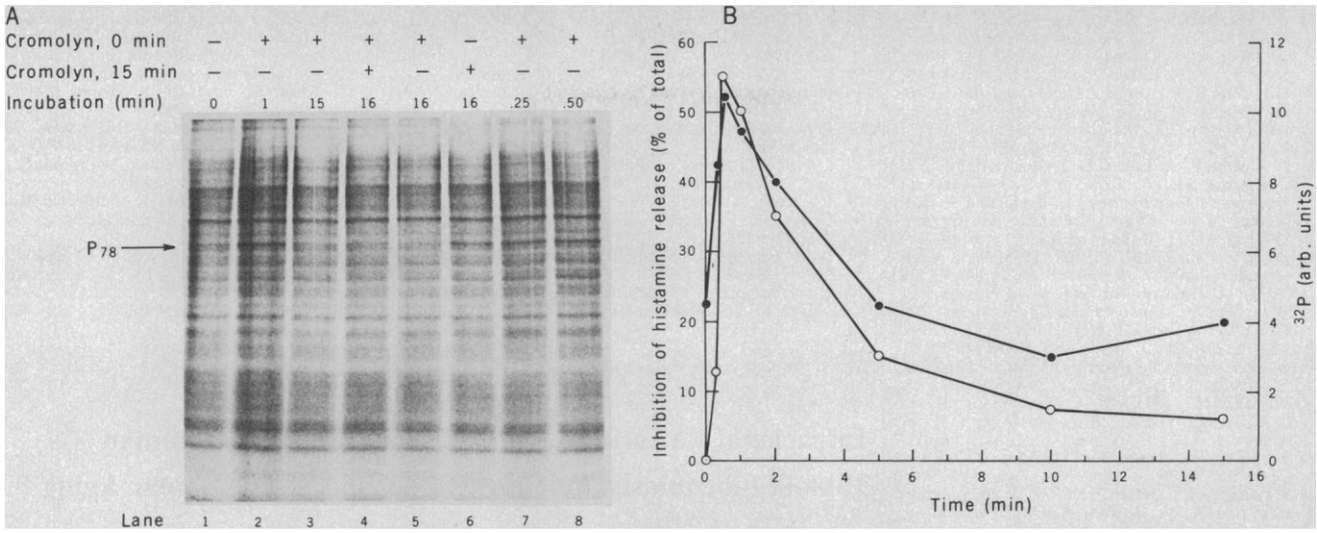


Fig. 2. (A) Autoradiograph showing the effect of cromolyn on incorporation of radioactive phosphate into the 78,000-dalton protein band as a function of time of incubation at 37°C; illustration of tachyphylaxis. Mast cells labeled with radioactive phosphate (as in Fig. 1) were incubated with cromolyn (10 $\mu M$) for 0, 0.25, 0.5, 1, or 15 minutes (lanes 1, 7, 8, 2, and 3, respectively). Some cells were incubated with cromolyn (10 $\mu M$) for 15 minutes, exposed to a second similar dose of cromolyn, and then examined 1 minute later (lane 4). Controls included cells incubated for 16 minutes, with cromolyn added at 0 minute (lane 5) or at 15 minutes (lane 6). Measurement of the amount of radioactive phosphate incorporated into the 78,000-dalton protein band yielded the following results (in arbitrary units): lane 1, 3.8; lane 2, 6.4; lane 3, 3.2; lane 4, 3.0; lane 5, 2.8; lane 6, 6.0; lane 7, 7.8; and lane 8, 9.0. (B) Comparison of the effect of cromolyn on incorporation of radioactive phosphate into the 78,000-dalton protein band and on histamine secretion from rat peritoneal mast cells, as a function of incubation time. To measure incorporation of radioactive phosphate (•), the reaction-stopping solution was added to samples that had been incubated with cromolyn (10 $\mu M$) for the indicated times, and protein phosphorylation was analyzed as described in the legend to Fig. 1. A similar time course was obtained in two other experiments. To measure histamine release (○), mast cells were incubated with cromolyn for the indicated times before terminating the reaction. Histamine release was induced in all tubes by adding 48/80 (1 $\mu$g/ml) 30 seconds before stopping the reaction. A similar time course was obtained in a second experiment.

course of the response to the secretagogue 48/80 (Fig. 1A). It is also evident in Fig. 1A that 48/80 led to increased incorporation of radioactive phosphate into three additional protein bands (68,000, 59,000, and 42,000 daltons). We believe that these protein bands, which are phosphorylated early in the response to 48/80, may be related to activation of the exocytotic secretory response (*10*). The concentration range over which cromolyn caused incorporation of radioactive phosphate into the 78,000-dalton protein band corresponded with that which inhibited histamine secretion induced by 48/80 (Fig. 1B); moreover, this concentration range is similar to the range over which cromolyn has been found to inhibit antigen-induced histamine release from sensitized rat mast cells (*15*). In experiments in which 48/80 was given at the same time as cromolyn, the phosphorylation of the 78,000-dalton protein seemed no less prominent (*16*).

Further evidence of correspondence between histamine release and protein phosphorylation was obtained from experiments in which we studied tachyphylaxis, a characteristic feature of the inhibitory effect of cromolyn on histamine secretion in mast cells (*7, 8*). This inhibitory effect, although pronounced when the secretagogue is given at the same time as cromolyn, fades rapidly so that little or no inhibition is evident when the secretagogue is given only a few minutes after cromolyn (*7, 8*) (Fig. 2B). Our experiments showed a parallel behavior with respect to phosphorylation. Thus the radioactive phosphate incorporated into the 78,000-dalton protein band and the inhibitory effect on histamine release disappeared at a similar rate (Fig. 2). Moreover, a second exposure to cromolyn after the effect of a first exposure had waned failed to induce incorporation of radioactive phosphate into the 78,000-dalton protein band (Fig. 2A, lane 4), just as it fails to inhibit histamine secretion (*7, 8*). This failure of cromolyn, on a second testing, could not be accounted for by the longer incubation of 15 minutes; when cromolyn was added for the first time after a 15-minute incubation, cells incorporated an increased amount of radioactive phosphate into this protein (Fig. 2A, lane 6).

These observations suggest that a protein with a molecular weight of about 78,000 may be involved in the mechanism that turns off or inhibits histamine secretion in mast cells. Since calcium influx or mobilization has been proposed as the factor that mediates the secretory response, it could be that the phosphorylation we observed is involved in the down regulation of calcium. It has been suggested (*9*) that cromolyn may facilitate closure of calcium gates in the mast cell membrane. From this point of view, phosphorylation of a 78,000-dalton protein might be a component of the mechanism of gate closure. Alternatively, phosphorylation of this protein might be involved in lowering intracellular free-calcium concentrations by promoting calcium sequestration; protein phosphorylation does seem to be involved in the sequestration of cytosolic free calcium in cardiac muscle (*17*) and in platelets (*18*).

The present results may not only offer a clue to the mode of action of a remarkable antiallergic drug, but may also provide some insight into the more general problem of the molecular events that terminate secretory responses.

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### References and Notes

1. D. Lagunoff, *Biochem. Pharmacol.* **21**, 1889 (1972); W. W. Douglas, *Biochem. Soc. Symp.* **39**, 1 (1974); B. Uvnäs, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 2172 (1974); A. Goth and A. R. Johnson, *Life Sci.* **16**, 1201 (1975); J. C. Foreman, L. G. Garland, J. L. Mongar, *Symp. Soc. Exp. Biol.* **30**, 193 (1976); H. Metzger, in *Immune Systems: Genetics and Regulation*, E. E. Sercarz, L. A. Herzenberg, C. F. Fox, Eds. (Academic Press, New York, 1977), p. 697; W. Kazimierczak and B. Diamant, *Prog. Allergy* **24**, 295 (1978).
2. R. R. Llinás and J. E. Heuser, *Neurosci. Res. Program Bull.* **15**, 557 (1977); W. W. Douglas, *Ciba Found. Symp.* **54**, 61 (1978).

3. W. W. Douglas, *Br. J. Pharmacol.* **34**, 451 (1968); B. Katz, *The Release of Neurotransmitter Substances* (Thomas, Springfield, Ill., 1969), pp. 1–59; P. F. Baker, *Recent Adv. Physiol.* **9**, 51 (1974); R. P. Rubin, *Calcium and the Secretory Process* (Plenum, New York, 1974), pp. 1–189.
4. G. L. Zink, *Am. J. Pharm.* **146**, 101 (1974); K. F. Austen, R. A. Lewis, D. J. Stechschulte, S. I. Wasserman, R. W. Leid, Jr., E. J. Goetgl, in *Progress in Immunology*, L. Brent and J. Holborow, Eds. (North-Holland, Amsterdam, 1974), p. 61; M. Kaliner and K. F. Austen, *Annu. Rev. Pharmacol.* **15**, 177 (1975); S. I. Wasserman, *Scand. J. Respir. Dis. Suppl.* **98**, 28 (1977); J. Padawer, in *Immediate Hypersensitivity: Modern Concepts and Developments*, M. K. Bach, Ed. (Dekker, New York, 1978), p. 301.
5. T. S. C. Orr, *Br. J. Dis. Chest* **67**, 87 (1973); C. E. Reed, *Med. Clin. North Am.* **58**, 55 (1974); K. F. Austen and R. P. Orange, *Am. Rev. Respir. Dis.* **112**, 423 (1975); N. A. M. Paterson, R. W. Leid, Jr., J. W. Said, S. I. Wasserman, K. F. Austen, in *Lung Cells in Disease*, A. Bouhuys, Ed. (Elsevier, Amsterdam, 1976), p. 223; R. A. Lewis and K. F. Austen, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 2676 (1977); L. M. Lichtenstein and K. F. Austen, *Asthma: Physiology, Immunopharmacology and Treatment* (Academic Press, New York, 1977), pp. 1–141.
6. J. S. G. Cox, in *Pharmacological and Biochemical Properties of Drug Substances*, M. E. Goldberg, Ed. (American Pharmaceutical Association, Washington, D.C., 1977), p. 277; R. G. Coffey and E. Middleton, in *Immunopharmacology*, J. W. Hadden, R. G. Coffey, F. Spreafico, Eds. (Plenum, New York, 1977), p. 203; A. F. Wilson and J. J. McPhillips, *Annu. Rev. Pharmacol. Toxicol.* **18**, 541 (1978); H. Johnson, in *Immediate Hypersensitivity: Modern Concepts and Developments*, M. K. Bach, Ed. (Dekker, New York, 1978), p. 533.
7. C. J. Falliers, *Pediatr. Clin. North Am.* **22**, 141 (1975); E. Middleton, Jr., *ibid.*, p. 111; D. C. Webb-Johnson and J. L. Andrews, *N. Engl. J. Med.* **297**, 476 (1977); *ibid.*, p. 758; M. Weinberger and L. Hendeles, *Postgrad. Med.* **61**, 85 (1977); *ibid.*, p. 958.
8. Several studies have found that 48/80-induced histamine secretion from rat peritoneal mast cells is inhibited by cromolyn in micromolar concentrations (median effective concentration, about 1 to 5 $\mu M$) similar to those reported (*16*) to inhibit histamine secretion induced by antigen from the same cells [H. G. Johnson and C. A. Van Hout, *Int. Arch. Allergy Appl. Immunol.* **50**, 446 (1976); H. G. Johnson, in *Immediate Hypersensitivity: Modern Concepts and Developments*, M. K. Bach, Ed. (Dekker, New York, 1978), p. 553; H. G. Johnson, C. A. Van Hout, J. B. Wright, *Int. Arch. Allergy Appl. Immunol.* **56**, 416 (1978)]. Our findings, depicted in Fig. 1, are in accord with this. Other studies have found that cromolyn is slightly less effective [see, for example, M. Chasin, C. Scott, C. Shaw, F. Persico, *ibid.* **58**, 1 (1979)] or much less effective [L. G. Garland and J. L. Mongar, *Br. J. Pharmacol.* **50**, 137 (1974); G. W. Read, M. Knoohuizen, A. Goth, *Eur. J. Pharmacol.* **42**, 171 (1977)] against 48/80. The variation in results may reflect, in part at least, differences in the intensity of the responses elicited by 48/80. It was noted by T. S. C. Orr, D. E. Hall, J. M. Gwilliam, and J. S. G. Cox [*Life Sci.* **10**, 805 (1971)] that the inhibitory effect of cromolyn against 48/80 was lost when the dose of 48/80 was increased to elicit large secretory responses. Additional causes of variation may be the use of purified or unpurified mast cells and also the peculiar bell-shaped dose-response effect of cromolyn in which inhibition tends to diminish as the cromolyn concentration is increased above a certain value (see H. G. Johnson, cited above).
9. J. C. Foreman and L. G. Garland, *Br. Med. J.* **1**, 820 (1976); J. C. Foreman, M. B. Hallett, J. L. Mongar, *Br. J. Pharmacol.* **59**, 473P (1977).
10. W. Sieghart, T. C. Theoharides, S. L. Alper, W. W. Douglas, P. Greengard, *Nature (London)* **275**, 329 (1978).
11. T. C. Theoharides and W. W. Douglas, *Endocrinology* **102**, 1637 (1978).
12. D. C. Morrison, J. F. Roser, P. M. Henson, C. C. Cochrane, *J. Immunol.* **112**, 573 (1974); R. H. Hino, C. K. H. Lau, G. W. Read, *J. Pharmacol. Exp. Ther.* **200**, 658 (1977).
13. P. Röhlich, P. Anderson, B. Uvnäs, *J. Cell Biol.* **51**, 465 (1971); M. Kagayama and W. W. Douglas, *ibid.* **62**, 519 (1974).
14. W. W. Douglas and Y. Ueda, *J. Physiol. (London)* **234**, 97P (1973); E. Woldemussie and N. C. Moran, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 603 (1978); J. H. Baxter and R. Adamik, *Biochem. Pharmacol.* **27**, 497 (1978).
15. L. G. Garland, *Br. J. Pharmacol.* **49**, 128 (1973); E. J. Kusner, B. Dubnick, D. J. Herzig, *J. Pharmacol. Exp. Ther.* **184**, 41 (1973).
16. Cromolyn did not block the 48/80-induced phosphorylation of the protein bands of molecular weights 68,000, 59,000 and 42,000.
17. P. V. Sulakhe and P. J. St. Louis, *Gen. Pharmacol.* **7**, 313 (1976); A. Wollenberger and H. Will, *Life Sci.* **22**, 1159 (1978); A. M. Katz, *Adv. Cyclic Nucleotide Res.* **11**, 303 (1979).
18. R. Kaser-Glanzmann, M. Jakabova, J. N. George, E. F. Luscher, *Biochim. Biophys. Acta* **466**, 429 (1977).
19. B. K. Krueger, J. Forn, P. Greengard, *J. Biol. Chem.* **252**, 2773 (1977).
20. It was important to keep the purified mast cells (which had been washed free of bovine serum albumin) on ice and to use them as quickly as possible because such cells rapidly lose their responsiveness to 48/80 at room temperature [K. Saeki, *Jpn. J. Pharmacol.* **14**, 375 (1964)].
21. L. T. Kremzner and I. B. Wilson, *Biochim. Biophys. Acta* **50**, 364 (1961).
22. Supported by PHS grants DA-01627, MH-17387, and NS-08440 to P.G. and NS-09137 to W.W.D.; W.S. was a Max Kade Fellow. We thank G. B. Eagleton for the gift of cromolyn.
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# Intraclonal Variation in Proliferative Potential of Human Diploid Fibroblasts: Stochastic Mechanism for Cellular Aging

Abstract. *At several points during the growth of a clone of human embryonic lung fibroblasts in vitro, 100 to 200 cells were removed at random and the proliferative potential of each cell was determined. At each sample point, a wide variation in remaining population doubling ability was observed among the individual cells and the distributions of doubling potentials were distinctly bimodal. Furthermore, the two cells arising from a single mitosis differed in their ability to proliferate by as many as eight population doublings (256-fold in the number of cells produced). The results suggest that a stochastic process is responsible for determining the limited proliferative potential of human embryonic lung fibroblasts.*

Normal human diploid fibroblasts have a limited life-span in vitro (that is, a finite proliferative potential in culture) (*1*). This potential has been shown to be inversely proportional to the age of the tissue donor (*2*) and has been widely studied as a model for cellular aging. Numerous biochemical and morphological changes occur as these cells reach the end of their proliferative potential (*1*). A number of hypotheses have been proposed to explain this phenomenon; however, direct biochemical analysis of cultures has not yet identified the mechanisms responsible for limiting the proliferative potential of normal human cells.

Many investigators (*3–6*) have reported heterogeneity in morphology, interdivision time, and ability for clonal growth among the individual cells of mass cultures of human diploid fibroblasts. At all stages in the life-span in vitro of human diploid fibroblast cultures (WI-38 cells), wide variation in the proliferative potential of isolated clones was observed (*7*). From these experiments, it was not possible to determine whether variability continues to develop during repeated doubling of the culture in vitro or whether the variation is due to heterogeneity among the individual cells in the tissue of origin. Alternatively, the variation might develop during the process of initiating the cells in culture. A more detailed investigation of this variation would provide some insight into the mechanisms determining proliferative potential. In addition, this information would provide a powerful data base against which to test hypotheses. In the experiments reported here, we found that heterogeneity in proliferative potential appears rapidly within a single clone of human diploid fibroblasts. We also found that the proliferative potentials of the two cells from a single mitotic event can differ by as many as eight population doublings (PD's).

To determine the intraclonal variation in proliferative potential, the distributions of doubling potentials were determined for three sets of subclones isolated from a single clone that was initiated from a culture of human embryonic lung cells (Flow 2000) (*8*) in vitro at PD 23. Two hundred cells were isolated at random from the parent clone at 16, 26, and 36 PD's after its initiation as a single cell (see legend to Fig. 1). Each subclone was observed until the limit of its replicative ability was reached, and frequency distributions of proliferative potential (the number of PD's achieved after isolation) were constructed for each set of subclones (Fig. 1). Cultures were considered to be at the end of their proliferative potential when they failed to double in cell number for 2 weeks. At PD 16, one of the subclones was used to initiate a second set of subclones, and the frequency distribution of their proliferative potential was determined (Fig. 1, inset).

Several characteristics of the distributions are noteworthy: (i) the parent clone was more homogeneous with respect to


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