Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, pp. 3478–3482, September 1974

Variants of Mouse Myeloma Cells That Produce Short Immunoglobulin Heavy Chains

(mutagenesis/immunoglobulin assembly/cultured cells)

BARBARA K. BIRSHTEIN, JEAN-LOUIS PREUD'HOMME*, AND MATTHEW D. SCHARFF

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Communicated by Harry Eagle, July 5, 1974

ABSTRACT **After mutagenesis of cultured mouse myeloma cells with ICR 191 or Melphalan, variant clones were isolated that synthesized immunoglobulin heavy chains shorter than those produced by the parent. These variants fell into two phenotypes, based on the size, serology, and pattern of assembly of the heavy chain. Variant chains of both types no longer reacted with antisera directed either against the Fc (C-terminal half of the heavy chains) or subclass-specific IgG_{2b} determinants. Comparative ion exchange chromatography of tryptic–chymotryptic peptides confirmed that the variant heavy chains differed structurally from those of the parent and from each other. A conversion from one phenotype to the other has been observed.**

Cultured mouse myeloma cells of the MPC-11 (IgG_{2b}) cell line spontaneously generate variants that have lost the ability to produce heavy chains, at a rate of 10^{-3}/cell per generation (1). Mutagenesis with the acridine mustard ICR 191 (2) or with the phenylalanine mustard Melphalan (3) can increase the incidence of variants 10- to 20-fold, so that they represent as many as 3–6% of the surviving clones. Most of the variants seen after mutagenesis are similar to those that arise spontaneously in that they have lost the ability to produce heavy (H) chains. However, some 20–30% of these mutagenized variants continue to synthesize H chains that differ structurally, serologically, and functionally from the parental heavy chain. Some of these H chains are smaller than the parental; others are larger than or equal in size to the parental chain. Here, we discuss those variants producing smaller H chains.

MATERIALS AND METHODS

The cell line 45.6 and its subclones 45.6.2.4 and 45.6.3.1 are all clones derived from the MPC-11 mouse myeloma tumor, which produces an IgG_{2b} immunoglobulin (4). The cells were grown suspended in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) supplemented with 20% horse serum, nonessential amino acids, glutamine, penicillin, and streptomycin. Mutagenesis was carried out by growing cells in the presence of 1 μg/ml of ICR-191† (2) or 0.4–0.6 μg/ml of Melphalan (3) (Burroughs Wellcome) for 24 hr, and then in medium lacking the mutagenizing agent for an additional 24 hr. Approximately 60% of the cells survived the ICR mutagenizing treatment and 20–30%, the Melphalan mutagenesis. The cells were cloned (7) in soft agar over feeder layers of rat embryo fibroblasts (Microbiological Assoc., Bethesda, Md.), and 3 days later were overlayed with agar containing antiserum against the Fc region (C-terminal half) of the MPC-11 molecule. In some experiments, antiserum against the completely reduced and alkylated MPC-11 H chain was used. Clones that secreted molecules containing normal H chains were obscured by the resulting antigen–antibody precipitate (1). Unstained clones (presumptive variants) were retrieved from the agar and inoculated into Linbro dishes (TS-FB-96) in conditioned medium. As cells increased in number, they were transferred to Linbro dishes (FB-16-24-TC) and then grown to mass culture in petri dishes (Falcon Plastics).

Variants were named in the following way: 45.6.2.4 ICR-11 was a variant clone picked after mutagenesis of the 45.6.2.4 cell line with ICR-191. 45.6.3.1 M-311 was a clone picked after mutagenesis of 45.6.3.1 cell line with Melphalan. Often, variants were recloned without further mutagenesis, e.g., 45.6 ICR 4.68. Only if subclones changed in phenotype were they given a new number, e.g., 45.6 ICR 4.68.7. The variants discussed in this paper have been numbered one through six in Table 1, and will be referred to by these numbers throughout the *text*.

Variants were characterized serologically by immunodiffusion analysis of cytoplasm and secretions. Three mls of a cell suspension (5 to 10 × 10^5 cells per ml) were centrifuged at 1000 rpm (International Portable Refrigerated Centrifuge model PR2) for 10 min to yield a cell pellet and supernatant (secretions). The cell pellet was resuspended in 0.05 ml of an isotonic buffer (0.01 M Tris, 0.15 M NaCl, and containing 8.5 ml 1 M HCl and 0.305 g $MgCl_2 \cdot 6H_2O$ per liter) containing 0.5% of the detergent Nonidet P-40 (NP-40), mixed to insure complete cell lysis, and then the nuclei were pelleted by centrifugation (2000 rpm–10 min) or by settling overnight in the cold. Antisera against the IgM and IgA classes and the IgG_1, IgG_{2a}, and IgG_{2b} subclasses were purchased from Meloy Laboratories and their specificity confirmed by testing them against various myeloma proteins. The MPC-11 protein was purified from the serum of animals bearing tumors by ammonium sulfate precipitation and chromatography on DEAE-cellulose (8) and followed on occasion by gel filtration on Sephadex G-200, or electrophoresis on acrylamide gels.

* Present address: Laboratoire d'Immunochimie, Université de Paris, Faculté de Médecine, Institut de Recherches sur les Maladies du Sang, Hôspital St.-Louis, 2, Place du Docteur-Fournier, Paris-Xe, France.

† ICR-191 was supplied by H. J. Creech of the chemotherapy laboratory of the Institute for Cancer Research, Philadelphia, Pa. The preparation and properties of ICR-191 and other ICR mutagens are described in refs. 5 and 6.

Antisera against the completely reduced and alkylated H chain, the Fab, or the Fc of the MPC-11 protein were raised by injecting rabbits repeatedly with antigen in complete Freund's adjuvant.

Immunoglobulin molecules were further analyzed by electrophoresis on acrylamide gels containing sodium dodecyl sulfate (9). First, variant clones were incubated with radioactive valine, threonine, and leucine (4), after which radiolabeled intracellular and secreted immunoglobulins were immunoprecipitated by means of an indirect technique (10). Five microliters of a high titer rabbit antiserum to the MPC-11 H and light (L) chains was added to the cell lysate (derived from NP-40 treatment) or to the secreted material from 2×10^6 cells and allowed to react for 1 hr or more at 4°. Then 0.1 ml of a sheep antiserum against rabbit IgG was added and allowed to react overnight at 4°. The resulting immunoprecipitate was washed by centrifugation through a 2-ml layer of 1 M sucrose in phosphate-buffered saline (0.15 M NaCl, 0.02 M sodium phosphate, pH 7.0), dissolved in 0.1–0.2 ml of 2% sodium dodecyl sulfate, and placed in a boiling-water bath for 1 min.

Reduction of disulfide bridges was carried out by treating samples in 2% sodium dodecyl sulfate with 0.15 M 2-mercaptoethanol in tightly closed tubes at 37° for 3 hr. Alkylation of the resulting sulfhydryl groups was done by adding enough 0.6 M iodoacetamide to make the concentration 0.18 M. Such samples were applied directly to 20 cm, 5% acrylamide gels containing 0.1 M sodium phosphate (pH 7) and 0.1% sodium dodecyl sulfate (9). Samples to be analyzed without separation of the component chains were treated with iodoacetamide twice: (*1*) before lysis of the cells with NP-40, iodoacetamide was added either to the cell pellet or to the cells suspended in isotonic buffer; (*2*) iodoacetamide was added to the immunoprecipitate before dissolution with sodium dodecyl sulfate. The final concentration of iodoacetamide in both cases was 0.06 M.

For routine analysis of labeled immunoglobulin, sodium dodecyl sulfate gels were fractionated by means of the Maizel Gel Grinder (Savant Instruments), and the radioactivity of individual fractions was determined either in a low background counter (Nuclear Chicago) or in a scintillation counter (Beckman) following the addition of 10 ml of Triton–toluene scintillation fluid (11) to each 1-ml sample of gel in water. Molecular weights were calculated from the relative migration of the labeled proteins on sodium dodecyl sulfate gels, assuming a molecular weight of 55,000 for the H chains and 23,000 for the L chains. Gels were also used as a preparative tool. Radiolabeled immunoglobulin components were eluted from the gel with 1% sodium dodecyl sulfate, aliquots were removed for radioactivity measurement, and pools were made according to the radioactivity profile.

For peptide analysis, ^{3}H-labeled parent and ^{14}C-labeled variant chains (or vice-versa) were mixed, and rabbit IgG (500 μg) was added as a carrier for precipitation of proteins with trichloroacetic acid (final concentration 10%). For removal of the sodium dodecyl sulfate, the proteins were washed successively with 5% trichloroacetic acid; 1:1, ethanol:diethyl ether; and diethyl ether. Samples were air-dried, resuspended in 2 ml of 0.05 M NH_4HCO_3, pH 8, and enzymatically digested by sequential additions of 0.5 mg of TRTPCK trypsin (Worthington) for 2 hr, 0.5 mg of chymotrypsin (Worthington) for 2 hr, and 0.5 mg of trypsin for 1 hr. Samples were freeze-dried and dissolved in 2 ml of 0.3 M pyridine, pH adjusted to 2.2 with HCl, and the peptides were resolved by ion exchange chromatography on a cation exchange resin (Spherix, type XX90, 7200, Phoenix Precision Instrument Co.) with a pyridine acetate gradient (linear gradient: 0.05 M pyridine, pH 3.1, to 2.0 M pyridine, pH 5.0).

RESULTS

Detection of Variants. Variants producing H chains different from those of the parent were easily found after mutagenesis with ICR-191 and Melphalan. In a representative experiment, clone 45.6.2.4 was treated with ICR-191 at a concentration of 1 μg/ml. Cells were cloned and overlayed with an antiserum against the Fc portion of the MPC 11 protein. Thirty of 821 (3.64%) clones were "unstained." These 30 presumptive variants were recovered from the agar and 19 were successfully grown to mass culture.

Ouchterlony analysis of cytoplasmic lysates showed that 11 clones contained only L chains and eight contained H and L chains. Four of the eight clones were found to resemble the

TABLE 1. *Variants of the MPC-11 cell line—the size and serological characteristics of the heavy chains they synthesize*

	Molecular weight of H chain	Cytoplasm				Secretion
		Anti-CRA§ H chain	Anti-Fab	Anti-Fc	Anti-γ2b	Anti-CRA H chains
Parent	55,000	+	+	+	+	+
Type 1	50,000	+ spur	+	−	−	−
1. 45.6 ICR 4.68*						
2. 45.6.2.4. ICR 7†						
3. 45.6.3.2. ICR 11†						
Type 2	40,000	+ spur	+	−	−	+
4. 45.6 ICR 4.68.7*‡						
5. 45.6.2.4. ICR 18†						
6. 45.6.3.1. M311*						

* Overlay antibody: anti-completely reduced and alkylated heavy chain. No. 4 is stained with this antiserum while no. 6 is not, although the secretions of both clones are detected by Ouchterlony analysis with the same antiserum. This observation indicates the relative insensitivity of the overlay technique, a point that has obviously favored us in our ability to select clone no. 6.

† Overlay antibody: anti-Fc.

‡ This clone was derived from no. 1 and is discussed in the text.

§ CRA means completely reduced and alkylated.

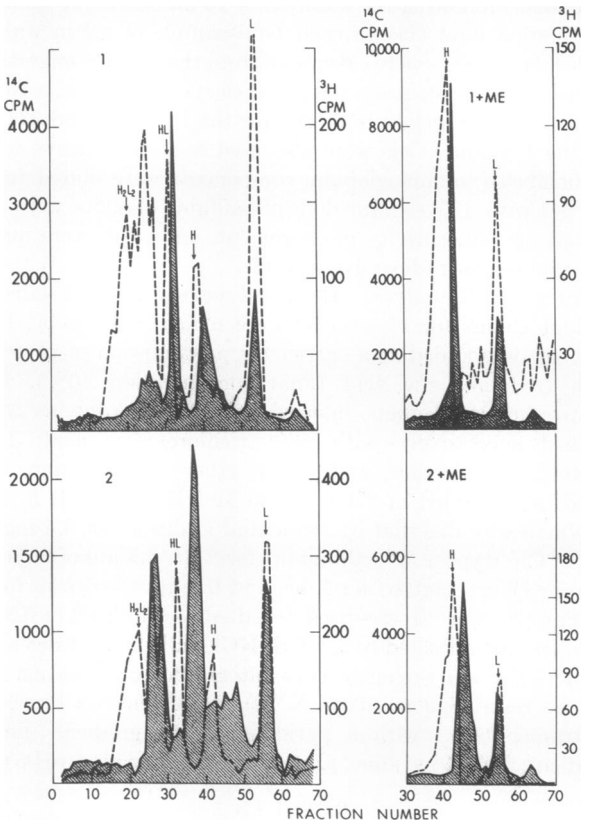

FIG. 1. Cytoplasmic immunoglobulin components of a Type 1 variant (number 1) and a Type 2 variant (number 4), each compared to the parent MPC-11 cell line. Cells were incubated with radioactive valine, threonine, and leucine for 10 min, after which immunoglobulin was specifically precipitated from the cytoplasmic lysate. The precipitates were submitted to electrophoresis on acrylamide gels containing sodium dodecyl sulfate. The details for these procedures are in the *Materials and Methods*. The shaded area marks the variants, the dotted line, the parent. The right hand panels show the cytoplasmic immunoglobulin after reduction of the disulfide bridges with 2-mercaptoethanol (ME).

parent in that they synthesized and secreted both H and L chains. They were considered to be mispicked and were discarded. Two of the clones containing H and L chains synthesized H chains smaller than the parent. The two remaining clones contained H chains of normal size. (J.-L. Preud'homme, B. K. Birshtein, and M. D. Scharff, manuscript in preparation.) The derivation of all clones described in this paper is listed in Table 1.

Two Groups of Variants. Six clones producing small H chains fell into two groups: Type 1, numbers 1, 2, and 3 in Table 1, each of which synthesized H chains of about 50,000 molecular weight; and Type 2, numbers 4, 5, and 6 in Table 1, each of which synthesized heavy chains of about 40,000 molecular weight. The assembly patterns of a representative of each type, numbers 1 and 4 in Table 1, are shown in Fig. 1. The right hand panels show the comparison of H and L chains in the parent and variant. The H chain of the Type 2 variant is smaller than that of the Type 1 variant, and both are smaller than the parent chain. The L chains are the same size in five of the six variants, and comparative peptide maps of two of these (numbers 1 and 6) are identical. In the sixth variant

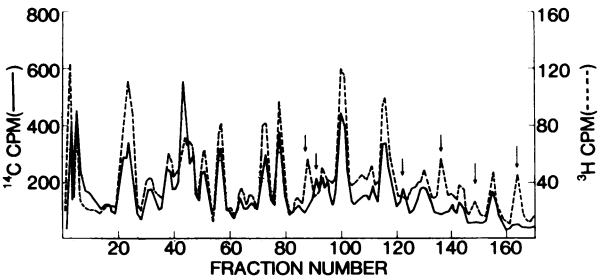

FIG. 2. Comparative peptide profiles of ^{14}C-labeled H chains of a Type 1 clone (number 1) (solid line) with ^{3}H-labeled H chains of parent 45.6.2.4., (broken line). The details of the procedures are found in the *text*. Arrows mark differences in the profiles.

(number 5), the L chains of the variant are smaller than those of the parent by one fraction.

After 10 min of incubation with radioactive valine, threonine, and leucine, both the parent and the Type 2 variant have assembled much of the newly synthesized H and L chains into polymers (lower left panel). In contrast, Type 1 clones seem unable to form inter-H chain disulfide bonds and are blocked at the HL stage of assembly.

Serological Characterization of Variants. Ouchterlony analysis of cell lysates and secretions with four antisera showed serological differences between the variants and the parent, and between the two types of variants (Table 1). A comparison of variants and parent with an antiserum against completely reduced and alkylated MPC-11 H chain (anti-CRA H chain) showed the variants to be lacking antigenic determinants included in the parent, as shown by a "spur" on the Ouchterlony plate. These missing antigenic determinants included those recognized by rabbit anti-Fc and rabbit anti-IgG_{2b}. The antigenic determinants recognized by rabbit anti-Fab were present in both variants. Although Type 1 variants secreted H chains, these secretions were unreactive with anti-CRA H chain, while the secretions of Type 2 variants reacted well with this antiserum. This observation provided an easy tool for distinguishing these types of variants.

Peptide Analysis of Variants. To determine whether the smaller size and loss of antigenic determinants by the short chain variants were associated with a change in their primary structure, we compared the H chains from a Type 1 Variant (number 1) and from a Type 2 variant (number 6), each with the parent H chain by ion-exchange chromatography of tryptic–chymotryptic peptides. Figs. 2 and 3 show that the profiles are strikingly similar but that of the Type 1 variant (Fig. 2) lacks four peaks found in the profile of the parent, and contains two small peaks not present in the parent profile. The Type 2 variant (Fig. 3) showed a similar profile but more differences were seen. These differences seem not to be due to isotopic shifts, since the profiles of ^{3}H- and ^{14}C-labeled parent H chain are identical (4). Comparison of tryptic peptides also showed differences, indicating that partial cleavages by chymotrypsin were not responsible for different profiles.

Variant number 1 was recloned twice because it rapidly generated spontaneous variants which synthesized only L chains and because at one point it had become contaminated with slow-growing microorganisms. Clone 45.6 ICR 4.68 was very much like the original variant, but when it was

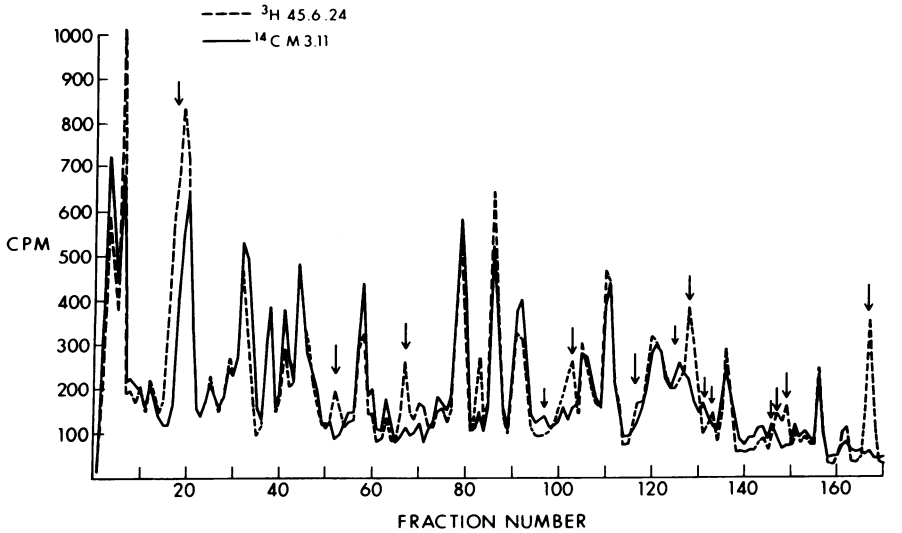

FIG. 3. Comparative peptide profile of ^{14}C-labeled H chains of a Type 2 clone (number 6) (solid line) with ^{3}H-labeled H chains of parent 45.6.2.4., (broken line). The details of the procedures are found in the *text*. Arrows mark differences in the profiles.

subcloned and overlayed with an antiserum directed against completely reduced and alkylated H chain, about one-third of the subclones were stained and the rest were unstained. Unstained variant clones either were making no H chains or were Type 1 variants. Stained clones now showed the Type 2 phenotype and synthesized a heavy chain of molecular weight approximately 40,000 (see footnote to Table 1). A comparison of the H chain from the Type 1 (number 1) and the Type 2 variant derived from it (number 4) is shown in Fig. 4. The larger chain contains all peptides seen in the smaller; the smaller lacks some four components (see arrows).

The same ICR Type 2 variant derived in this recloning experiment (number 4) was compared to a Melphalan Type 2 variant (number 6). The two peptide profiles are identical with the possible exception of one or two small peaks.

Preliminary labeling experiments of a representative of each type of variant (nos. 1 and 6) with radioactive fucose, glucosamine, galactose, and mannose showed that the variant H chains contain carbohydrate, but we do not yet know if the size and location of the carbohydrate moieties are the same as in the parent H chain.

DISCUSSION

Although a number of presumed mutations in cultured animal cells have been described, the putative mutant gene products are usually present in such small amounts that it has been difficult to define precise primary structural changes. This difficulty and the fact that a change in ploidy (12, 13) did not result in the expected change in mutation rates have led some investigators to question whether the presumed mutants are really the results of changes in the base sequence of the responsible structural genes. However, a number of investigators have described variants that seem to be the result of mutations (14–17). Of special relevance to this report, Secher *et al.* have described spontaneously occurring variants from mouse myeloma cells (18).

By the usual criteria, the variant heavy chains described in this paper are the products of mutant genes. The incidence of such variants is greatly increased by mutagenesis with some, but not with all, agents; the expression of the mutant phenotype usually remains stable in culture; the aberrant H chains are functionally and serologically different from the parental H chain; and, most importantly, the variant H chains have been shown to differ structurally from the parental H chain and from each other. Although we have not yet determined the exact amino acid sequence changes responsible for the lesion in the mutant protein, the lack of reactivity with anti-Fc and anti-IgG$_{2b}$ suggests a deletion somewhere in the C-terminal half of the H chain. Preliminary analysis of the cyanogen bromide fragments indicated that the normal Fc is absent in the variant. A deletion could be at the C-terminal end, perhaps caused by a frameshift that results in a nonsense mutation and a premature termination. Alternatively, there could be an internal deletion. Secher *et al.* have recently described two spontaneously occurring variants of the P$_3$ (MOPC-21) cell line; one of the abnormal proteins lacks the C-terminal domain, and the other has an internal deletion (18).

The results reported here raise a number of interesting questions about the genetic control of immunoglobulin synthesis in the cells under study: (*1*) Why do independent mutagenizing events lead to the same cast of variants? While the repeated isolation of the same types of variants could be the result of the techniques used to detect them, it may

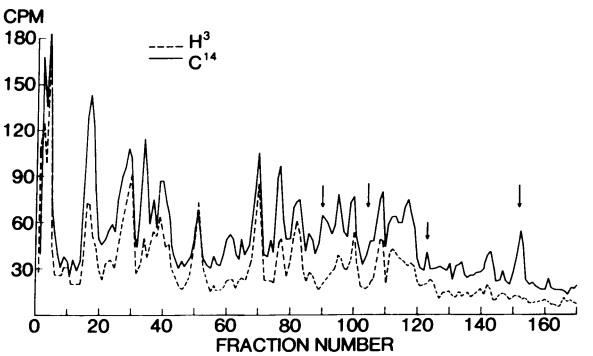

FIG. 4. Comparative peptide profile of ^{14}C-labeled H chains of a Type 1 clone (number 1) (solid line) with ^{3}H-labeled H chains of a Type 2 clone (number 4) (broken line). The details of the procedures can be found in the *text*. Arrows mark differences in the profiles.

also reflect a genetic hot spot or a resulting selective growth advantage of the particular variants. (*2*) What is the mechanism for the spontaneous conversion of type-1 to type-2 variants? Does the primary mutagenic event disturb a control mechanism that can then be jarred by conditions of cell culture or cloning? (*3*) Finally, the extremely high incidence of variants continues to be the most provocative aspect of these studies. The high incidence seems not to be related to the generation of antibody diversity, since we have no evidence for changes in the variable region of the H chain. Perhaps it could reflect something unusual in the biochemical genetics of the multigene system responsible for immunoglobulin production. In addition, the high incidence of variants also raises the possibility that the variant proteins are not the result of mutations in the structural genes. For example, they could arise from normally silent genes which for some reason have been turned on by ICR 191 or Melphalan. We hope that solutions to these questions can be achieved by a detailed structural characterization of the mutant gene products.

We thank Dr. Reuben Baumal for his initial isolation of variant 1 and Ms. Rochelle Epstein for her early studies on this variant. We also thank Ms. Lucille Frank and Ms. Theresa Jasek for their expert technical assistance. This work was supported by funds from the National Institutes of Health (AI 5231, AI 10702), the National Science Foundation (GB 29560), and the American Cancer Society. B.K.B. was a fellow of the New York Heart Association. J.L.P. was supported by the French National Institutes of Health Medical Research (INSERM) and a National Institutes of Health John E. Fogarty International Fellowship (1 FO 5TW-1862).

1. Coffino, P. & Scharff, M. D. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 219–223.
2. Baumal, R., Birshtein, B. K., Coffino, P. & Scharff, M. D. (1973) *Science* **182**, 164–166.
3. Preud'homme, J.-L., Buxbaum, J. & Scharff, M. D. (1973) *Nature* **245**, 320–322.
4. Laskov, R. & Scharff, M. D. (1970) *J. Exp. Med.* **131**, 515–541.
5. Creech, H. J., Breuninger, E., Hankwitz, R. F., Jr., Polsky, G. & Wilson, M. L. (1960) *Cancer Res.* **20** (Part 2), 471–494.
6. Peck, R. M., Preston, R. K. & Creech, H. J. (1961) *J. Org. Chem.* **26**, 3409–3414.
7. Coffino, P., Baumal, R., Laskov, R. & Scharff, M. D. (1972) *J. Cell. Physiol.* **79**, 429–440.
8. Potter, M. (1967) *Methods Cancer Res.* **2**, 105–157.
9. Maizel, J. V., Jr. (1971) in *Methods in Virology*, eds. Maramorosch, K. & Koprowski, H. (Academic Press, New York), pp. 179–246.
10. Horwitz, M. S. & Scharff, M. D. (1969) in *Fundamental Techniques in Virology*, eds. Habel, K. & Salzman, N. P. (Academic Press, New York), pp. 297–315.
11. Patterson, M. S. & Greene, R. C. (1965) *Anal. Chem.* **37**, 854–857.
12. Harris, M. (1971) *J. Cell. Physiol.* **78**, 177–184.
13. Metzger-Freed, L. (1972) *Nature New Biol.* **235**, 245–246.
14. Beaudet, A. L., Roufa, D. J. & Caskey, C. T. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 320–324.
15. Sharp, J. D., Capecchi, N. E. & Capecchi, M. R. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3145–3149.
16. Chan, V. L., Whitmore, G. F. & Siminovitch, L. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3119–3123.
17. Thompson, L. H., Harkins, J. L. & Stanners, C. P. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3094–3098.
18. Secher, D. S., Cotton, R. G. H. & Milstein, C. (1973) *FEBS Lett.* **37**, 311-316.