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Separation and Translation of the mRNAs Coding for α and β Chains of Rabbit Globin

(L-*O*-methylthreonine/*in vitro* protein synthesis/tryptic peptides)

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ABSTRACT **A method is presented to separate the mRNAs coding for α and β globins of rabbit reticulocytes. 10S RNA was extracted from the light and heavy polysomes created by incubation of reticulocytes with L-*O*-methylthreonine, and assayed for mRNA activity in an ascites cell-free extract. Tryptic digests of the *in vitro* products demonstrated that the heavy polysomes yielded β globin mRNA at least 90% free of α mRNA activity, and that the light polysomes yielded α mRNA at least 70% free of β mRNA activity.**

Sequence studies and functional analysis of mRNA from several bacteriophages have implicated secondary structure and the untranslated regions of a given messenger RNA (mRNA) in control of translation (1–3). Knowledge of untranslated sequences in eukaryotic mRNAs might be helpful in answering important questions about the control of protein synthesis in higher cells. Sequence studies on eukaryotic mRNA have been hampered by the unavailability of single eukaryotic mRNA species in sufficient purity and quantity.

Mammalian reticulocytes, which can be obtained in large quantities from anemic animals without significant contamination by other cell types, synthesize α and β globin almost exclusively (about 90%) (4) and can be used as a source of the two mRNAs encoding these proteins. Mixed (α and β) mRNA prepared from reticulocyte polysomes is translatable in various heterologous cell-free extracts (5–8). Unequivocal separation and translation of the *individual* mRNAs for the α and β chains have, however, not been reported. We report here the purification and translation *in vitro* of the mRNA for the β chain of rabbit globin. The procedure makes use of the finding of Rabinowitz *et al.* (9), that L-*O*-methylthreonine (MeThr) causes separation of the polyribosomes engaged in synthesis of α and β globin.

MATERIALS AND METHODS

D, L-*O*-methylthreonine was obtained from Cyclo Chemicals Co. and L-*O*-methyl-threonine from Sigma.

Solutions. *A:* 140 mM NaCl–5 mM KCl–1.5 mM $MgCl_2$. *B:* Complete incubation mix, as described by Rabinowitz *et al.* (9). *C:* 2 mM $MgCl_2$–1 mM S_2 Threitol. *D:* 1 mM S_2 Threitol–10 mM KCl–1.5 mM $MgCl_2$–10 mM Tris·HCl (pH 7.4). *E:* 100 mM NaCl–0.5% sodium dodecyl sulfate–1 mM EDTA–10 mM Tris·HCl (pH 7.4).

Abbreviations: MeThr, *O*-methylthreonine; S_2Threitol, dithiothreitol.

Preparation of Cells. 2.3–3.2 kg New Zealand rabbits were made anemic by subcutaneous injections of 2.5% phenylhydrazine, 1.0, 0.8, 0.6, 0.6, 0.8, and 1.0 ml, respectively, for 6 days. Each rabbit was bled on the eighth day from the ear, then by cardiac puncture into 20 ml of cold solution *A* containing 250 units of Heparin. The cells were washed three times with solution *A*. The reticulocyte count was 80–90%. All subsequent procedures were performed at 3°, except where indicated otherwise.

Synchronization with NaF. The cells were incubated with 4 volumes of solution *B*, plus 5.0 mM NaF for 45 min at 37°, then washed five times with 5 volumes of cold solution *A*, incubated at 0° for 1 hr in solution *A*, then washed again with solution *A*.

Treatment with MeThr. Synchronized cells were incubated with 4 volumes of solution *B* plus 28 mM L-MeThr or 58 mM D, L-MeThr for 1 hr at 0°, then for 40 min at 37°. The reaction was stopped by the addition of 5 volumes of cold solution *A*.

Preparation of Polysomes. Pelleted cells were lysed by the addition of 1.5 volumes of solution *C*. The lysate was cleared twice by centrifugation at 12,000 × *g* for 15 min, layered over 7 ml of 40% sucrose in solution *D*, and centrifuged in a Spinco Type 30 rotor at 28,000 rpm for 180 min. This centrifugation enriches the heavy polysomes in the resulting pellet. The pellet was gently resuspended in solution *D* to give a final concentration equivalent to 10–15 ml of cleared lysate per 1 ml of suspension. 0.05 ml of the suspension was diluted to 1 ml with solution *D* and applied to a single gradient. 1 ml (undiluted) was applied to each of several additional preparative (37.5 ml) 15–30% exponential sucrose gradients in solution *D* (made with 36 ml of 15% sucrose solution in the mixing chamber and 40% sucrose in the reservoir) (10). The gradients were centrifuged at 26,000 rpm for 200 min in the Spinco SW27 rotor. Only the gradient with 0.05 ml of suspended pellet was analyzed spectrophotometrically (Fig. 1) and, with the aid of the absorbance profile, the desired α-rich (*A*) fractions (peaks 2–3 counting from top to bottom of the tube) and β-rich (*B*) fractions (peaks 8–10) were removed with a pipette from the remaining preparative gradients.

Refractionation of Polysomes. Separated fractions were centrifuged in a Spinco type-30 rotor at 28,000 rpm for 20 hr. The pelleted polysomes were resuspended in solution *D* and again fractionated on 15–30% sucrose gradients, together

with a marker gradient to locate the fractions to be removed from the preparative gradients.

Isolation of 10S RNA. Appropriate fractions were pooled, made to 0.1 M NaCl–2 mM EDTA–and 0.5% sodium dodecyl sulfate, and precipitated with 3 volumes of ethanol for 8 hr at −20°. Precipitates were washed once each with ethanol and ether, dried at reduced pressure at 3°, then resuspended in solution *E* to give a total volume of 1 ml for each of the α and β samples (up to 200 A_{260} units/ml). The samples were warmed to 37° for 5 min; 1 ml was layered on 37.5 ml of 15–30% exponential sucrose gradients in solution *E* (7), and centrifuged at 26,000 rpm for 20 hr at 22° in the SW27 rotor. Fractions from the 7–12S region were collected, made 0.3 M in NaCl, and precipitated with 3 volumes of ethanol at −20°. The 10S RNA was further purified by a second fractionation on a 15–30% exponential sucrose gradient. The purified RNA was precipitated, washed three times with ethanol and once with ether, dried under reduced pressure at 3°, and dissolved in water at 1 mg/ml.

Preparation of unseparated mRNA involved centrifugation of twice-cleared lysate over 7 ml of 36% sucrose in solution *D* in the Type-30 rotor at 28,000 rpm for 240 min at 3° (5, 7). The resulting polysome pellet was resuspended in solution *E*, and the 9–10S RNA was purified by the same methods used for the separated mRNAs.

Cell-Free Translation. Messenger function of the isolated RNAs was analyzed in a cell-free extract from Krebs II ascites tumor, according to Housman *et al.* (7). A typical 150 μl reaction contained per ml; 35 μg of 9–10S RNA, where indicated; 30 μmol HEPES (Hydroxyethylpiperazine ethanesulfonic acid), pH 7.0; 5 μmol Mg (acetate)$_2$; 1.5 μmol ATP; 0.3 μmol GTP; 550 IU creatine phosphokinase; 10 μmol creatine phosphate; 75 nmol each of 20 amino acids minus the labeled amino acid; 65 μCi of [^{3}H]tyrosine or 5 μCi of [^{35}S] methionine, and 650 μl of ascites S-30. The samples were incubated for 40 min at 37°; the solution was then made 2 mM

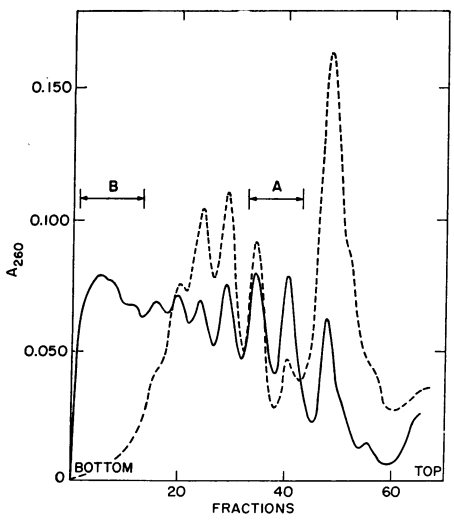

FIG. 1. Effect of MeThr on polysome profile. Cells were incubated with (——) or without (– – –) MeThr. The polysome pellets were resuspended and centrifuged on 15–30% exponential sucrose gradients at 26,000 rpm for 200 min at 3° in an SW27 rotor. *Brackets* indicate the fractions taken for *A* (α-rich) and *B*(β-rich) polysomes.

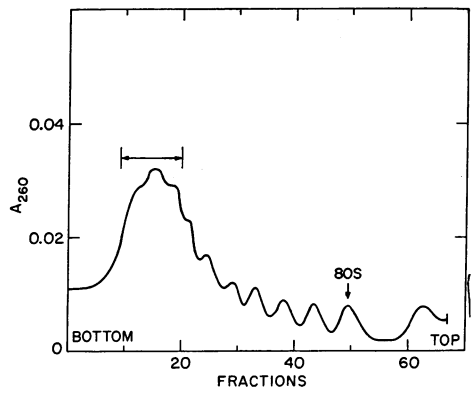

FIG. 2. Refractionation of β polysomes. Fractions from the first polysome gradient (Fig. 1) that contained β polysomes were pooled. The polysomes were pelleted and recentrifuged on a 15–30% exponential sucrose gradient. *Brackets* indicate the fractions taken for β polysomes.

in EDTA. Pancreatic RNase was added to a concentration of 50–100 μg/ml and the solution was further incubated at 37° for 5 min. The samples were precipitated with 5% Cl_3-CCOOH, carrier globin labeled with [^{3}H]methionine or [^{14}C]tyrosine—as appropriate—was added, and the samples were washed twice with 5% Cl_3CCOOH, digested with trypsin, lyophilized twice, and subjected to electrophoresis on paper at pH 3.5 for methionine-labeled peptides, and pH 6.5 for tyrosine-labeled peptides in pyridine–acetate buffer. 1-cm Strips were cut from the electrophoresis paper, eluted, and counted (7).

RESULTS AND DISCUSSION

Rabbit α-globin chains contain three isoleucine residues, which are located, respectively, 10, 17, and 55 aminoacid residues from the amino terminus, whereas β chains have a single isoleucine residue at position 110 (12). Incubation of rabbit reticulocytes with L-MeThr, a competitive inhibitor of isoleucine aminoacyl synthetase, causes a redistribution of ribosomes in the polysome profile. Untreated reticulocyte polysome preparations show predominantly polysomes of 4–5 ribosomes (Fig. 1). In the presence of MeThr, polysomes of 2–3 and 7–10 ribosomes predominate (9). Kazazian and Freedman have demonstrated that nascent polypeptide chains associated with the larger polysomes in MeThr-treated cells are predominantly nascent β chains, while the smaller polysomes carry predominantly nascent α chains (13).

We modified the procedures of Kazazian and Freedman in several respects, to improve separation of the α and β mRNA. Cells were incubated in 5 mM NaF for 45 min before incubation with MeThr. NaF is a specific, but reversible, inhibitor of the initiation of protein synthesis in rabbit reticulocytes (14, 15). Because elongation is not inhibited by NaF, ribosomes that are translating the α mRNA "run off" from polysomes before MeThr is added, thereby decreasing the number of ribosomes that have proceeded beyond the second and third isoleucine residues of the α chain. We also repurified regions of the gradient containing polysomes of 7–10 ribosomes by an additional centrifugation.

Average polysome profiles, with and without MeThr, are shown in Fig. 1; these results are comparable to the MeThr effect first observed by Rabinowitz *et al.* (9). The effective-

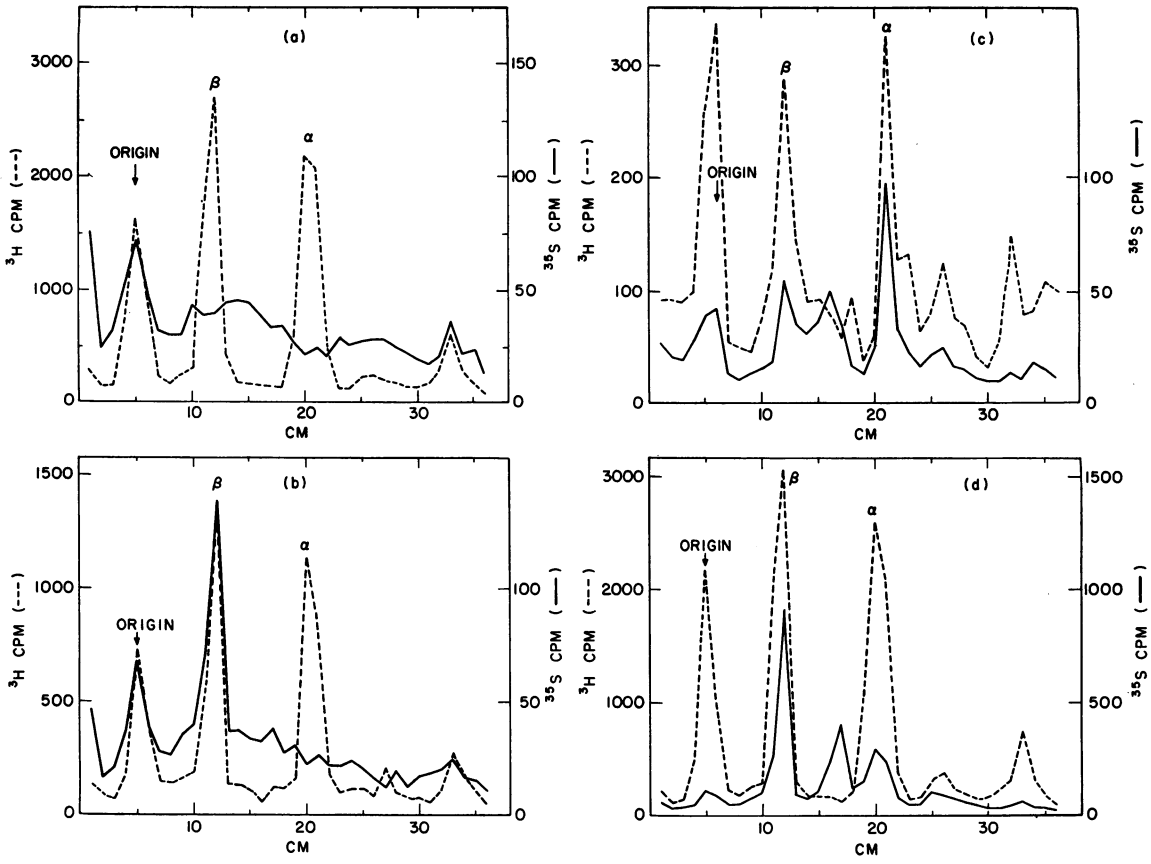

FIG. 3. Electrophoresis of methionine tryptic peptides from the product on the *in vitro* assay. The methionine-labeled products (——) were digested with trypsin and subjected to electrophoresis in pyridine–acetic acid buffer, pH 3.5; 1-cm strips were eluted and counted (7, 10). Marker [^{3}H]methionine-labeled globin (– – –) was added immediately before digestion with trypsin. (*a*) No added mRNA; (*b*) plus β mRNA; (*c*) plus α mRNA; (*d*) plus mixed mRNA.

ness of the MeThr in separating the α and β polysomes was somewhat variable among animals, and sometimes from one bleeding to the next. Synchronization with NaF improved the separation by lowering the amount of A_{260} in the polysome region of 5–7 ribosomes (data not shown), but this diminution is probably not required for adequate separation of the two polysome populations. In experiments reported here, only the β polysomes were centrifuged and fractionated a second time, as shown in Fig. 2. This procedure appeared to cause little polysome breakdown or runoff, and allowed improved separation of the 7–8 ribosome region.

To assay the relative purity of the separated mRNAs, the RNA was translated (7) in a cell-free extract of Krebs II ascites cells. Labeled globin, with a known α to β ratio of 1, was added to the completed reaction as a control for selective chain losses; the mixture was digested with trypsin and subjected to paper electrophoresis.

The α and β chains of rabbit globin each contain a single methionine residue, and together these yield two methionine-containing tryptic peptides, α T_5 and β T_5. Fig. 3*d* shows the results of adding unfractionated globin mRNA to the cell-free extract. As noted by others (8, 16), the α to β ratio (α T_5/β T_5) of the product was less than 1, and in this case was about 0.4. Also, this particular profile contained an additional peak at 17 cm that has not been characterized. Fig. 3*b* presents the digest pattern with added β mRNA. Nearly all the radioactivity above background (Fig. 3*a*) migrated coincidentally with the marker peptide β T_5. There was no detectable radioactivity above background at the position of α T_5, indicating that the purified β mRNA contained less than 10% α mRNA activity. Interpretation of the results of addition of α mRNA (Fig. 3*c*) is obscured by the high background in the β region of the blank (Fig. 3*a*).

Tyrosine-labeled tryptic digests of products of unseparated mRNA (Fig. 4*d*) yielded a pattern characteristic of globin produced by intact cells. There are three tyrosine peptides in each globin chain, and these appear to have been synthesized in about normal relative amounts for each chain; here also, the α to β ratio is less than 1. β mRNA (Fig. 4*b*) directs synthesis of peptides that comigrate with the β peptides synthesized by intact cells. Again the ratio of β peptides to α peptides syntheized was greater than 10:1. The products stimulated by α mRNA (Fig. 4*c*) gave a pattern of predominantly α peptides. All the β peptide positions did, however, contain small amounts of radioactivity, suggesting 30% or less β contamination of the α mRNA preparation. In addition, there was an unidentified labeled peptide(s) at 40–47 cm. Preliminary experiments suggest that repeated fractionation of the α polysome population can improve the separation of the α and β mRNAs.

With the methods described here, about 1 mg of 9–10S RNA was obtained from 80 ml of packed cells; the yield of either of the separated mRNAs was about 200 μg. The methods offer, therefore, an efficient means of isolating a single mRNA highly purified from other mRNAs, and in yields probably adequate for sequence studies. The purified

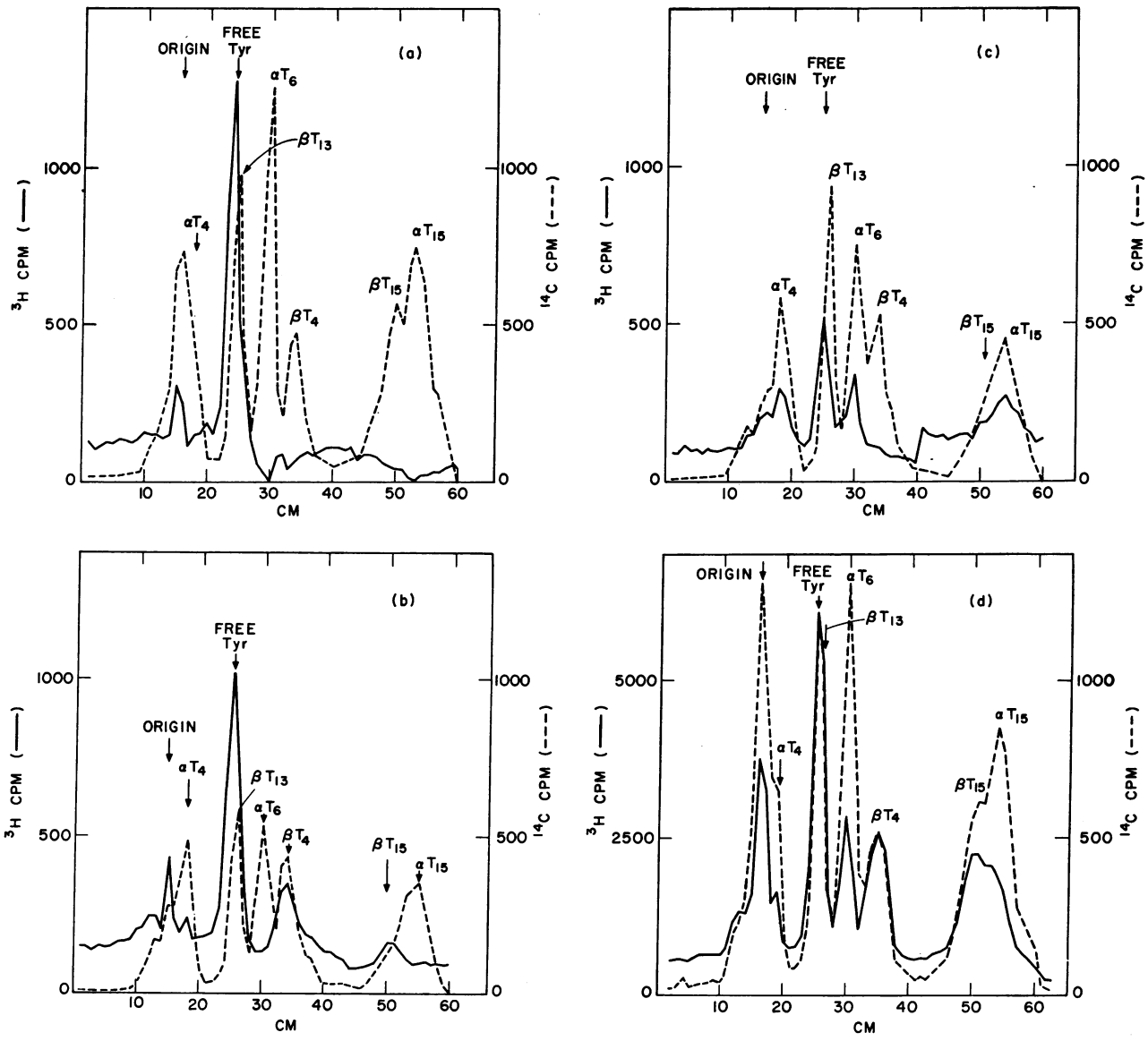

FIG. 4. Electrophoresis of tyrosine tryptic peptides from the product of the *in vitro* assay. The tryosine-labeled products were digested with trypsin and subjected to electrophoresis at pH 6.5; 1-cm strips were eluted and counted. Marker [^{14}C]tyrosine-labeled globin was added immediately before digestion with trypsin. (*a*) No added mRNA; (*b*) plus β mRNA; (*c*) plus α mRNA; (*d*) plus mixed mRNA.

β mRNA appears to have been translated faithfully *in vitro*, since the tryptic peptide patterns are nearly identical to those of β globin produced by intact cells.

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1. Steitz, J. A., Dube, S. K. & Rudland, P. S. (1970) *Nature* **226**, 824–827.
2. Adams, J. M. & Cory, S. (1970) *Nature* **227**, 570–574.
3. Lodish, H. F. & Robertson, H. D. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 655–673.
4. Bulova, S. I. & Burka, E. R. (1970) *J. Biol. Chem.* **245**, 4907–4912.
5. Lockard, R. & Lingrel, J. (1969) *Biochem. Biophys. Res. Commun.* **37**, 204–212.
6. Gurdon, J. B., Lane, C. D., Woodland, H. R., & Marbaix, G. (1971) *Nature* **233**, 177–182.
7. Housman, D., Pemberton, R. & Taber, R. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2716–2719.
8. Mathews, M. B., Osborn, M. & Lingrel, J. B. (1971) *Nature New Biol.* **233**, 206–209.
9. Rabinowitz, M., Freedman, M. L., Fisher, J. M. & Maxwell, C. R. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 567–578.
10. Noll, H. (1967) *Nature* **215**, 360–363.
11. Housman, D., Jacobs-Lorena, M., Rajbhandary, U. L. & Lodish, H. F. (1970) *Nature* **227**, 913–918.
12. Dayoff, M. O. & Eck, R. V. (ed.) (1969) "Atlas of protein sequence and structure," *Nat. Biomed. Res. Found.*, pp. 128 & 142.
13. Kazazian, H. H. & Freedman, M. L. (1968) *J. Biol. Chem.* **243**, 6446–6450.
14. Marks, P. A., Burka, E. R., Conconi, F. M., Perl, W. & Rifkind, R. A., (1965) *Proc. Nat. Acad. Sci. USA* **53**, 1437–1443.
15. Lin, S., Mosteller, R. D. & Hardesty, B. (1966) *J. Mol. Biol.* **21**, 51–69.
16. Nienhuis, A. W., Laycock, D. G. & Anderson, W. F. (1971) *Nature New Biol.* **231**, 205–208.