Immunological Characterization of Rabbit Hemoglobin α and β Chain-synthesizing Polysomes

ESTIMATION OF RELATIVE NUMBERS OF ACTIVE α- AND β-MESSENGER RIBONUCLEIC ACID*

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SUMMARY

Anti-hemoglobin chain antibodies, purified by immunoadsorption to Sepharose-linked antigen and thereby made both RNase-free and immunologically specific for either β or α chain, are readily bound by nascent hemoglobin chains attached to rabbit reticulocyte polysomes. Antibodies to other antigens, e.g. anti-immunoglobulin G, are not bound. Use of chain-specific antibodies enabled us to determine quantitatively the mean number of ribosomes associated with rabbit hemoglobin β- and α-messenger RNA. Such mean sizes of β- and α-polysomes could be calculated, for example, from density gradient profiles of polysomes previously coated with either [3H]isoleucine and [14C]valine. All estimates were similar; viz. the β:α ratio for mean number of ribosomes per mRNA ranged from 1.5 to 1.86. Among unaltered polysomes from the same rabbits, the β:α ratio for total relative numbers of synthetically active β and α ribosomes was calculated from Y intercepts of Dintzis-Naughton plots to be ~1.04 to 1.17. With these two kinds of β:α ratios as a basis, the relative numbers of active α-mRNA and β-mRNA were found to be somewhat greater than previously described by Lodish and Jacobsen (1972) J. Biol. Chem. 247, 3622). For individual animals, the α-mRNA:β-mRNA ratio ranged from 1.28 to 1.78. The addition of further 25% of α-mRNA which is unattached to polysomes (JACOBS-LORENA, M., AND BAGLIONI, C. (1972) Proc. Nat. Acad. Sci. U. S. A. 69, 1425) suggests that total α-mRNA is 2-fold more abundant than β-mRNA.

Immunological methods have recently been applied by Schimke and his associates (1-4) to the characterization and purification of hen oviduct polysomes which synthesize ovalbumin. The approach is straightforward; viz. antibodies developed against a completed polypeptide can react with nascent chains, i.e. species of polypeptide still attached to polysomes. The antibodies thus bound may be isolated by either immunoprecipitation or immunoadsorption and thereby messenger RNA for a particular polypeptide separated from other forms of mRNA. Although the same notion has occurred to many in the past (see Ref. 4 for brief review), Schimke and his colleagues were the first to bring analytical rigor to the procedure and its products.

In this report we apply immunological methods to the characterization of rabbit hemoglobin α and β chain-synthesizing polysomes and describe, first, the preparation and properties of the requisite immunological reagents; second, the binding between antibodies and reticulocyte polysomes, including, as a result, direct evidence that α polysomes (as previously supposed (5-7)) are considerably smaller than β polysomes; and, third, some derivative calculations indicating that the numbers of active α-mRNA exceed β-mRNA by a factor of at least 1.28 to 1.78. Elsewhere (8), we use the latter result to create an enlarged view of ways in which biosynthesis of hemoglobin α and β chains and their evolutionary changes are interrelated.

EXPERIMENTAL PROCEDURES

Materials and Methods

Chemicals—Reagent grade chemicals were used throughout. Rabbit, human, and goat immunoglobulin G were obtained from Mann Research Laboratories, Inc., Miles Laboratories, Inc., and Nutritional Biochemicals Corp.; CM-cellulose (CM32, microgranular standard) from Whatman; Sepharose 2B and Sephadex from Pharmacia Fine Chemicals, Inc. All enzymes used were obtained from Worthington Biochemical Corp., including trypsin reacted with L-1-tosylamido-2-phenylethyl chloromethyl ketone to inactivate chymotryptic activity. Radioactive amino acids and radionuclides were purchased from New England Nuclear Corp. and Amersham-Searle.

Sterile Technique—Adventitious RNase contamination was minimized through the use of acid-washed autoclaved glassware, sterile plastic pipettes, acid-washed plastic tubes, and autoclaved solvents; when practical, plastic gloves were worn in all maneuvers which involved materials ultimately in contact with polysomes. Early in our studies, sucrose solutions were prepared from ultrapure sucrose crystals (Mann) or sterile 55% sucrose solution (Mann). In later experiments, sucrose, Sepharose and all non-

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protein solutions were sterilized (9) with diethylpyrocarbonate (Eastman Kodak Co.), using ~0.1 ml per liter of solution.

Assay of Protein Concentration and Composition—Aliquots of protein or peptide solutions hydrolyzed in HCl were assayed for amino acid composition as previously described (10). Amounts of whole protein or polypeptide were calculated from sums of individual amino acids, omitting tryptophan and cysteine; molar quantities were deduced from molecular weights (11).

Measurement of Radioactivity—Radioactivity was assayed (10) in either a Bray's solution or, when in polysomes, in gels containing 5 ml of water and 5 ml of Aquasol (New England Nuclear). 125I and 3H were measured with ~60% efficiency.

Ribonuclease Assay—Hydrolysis of synthetic poly(U) (Miles Laboratories) was used as a gauge of RNase activity in supernatant or derived proteins. After incubation, supernatants remaining after precipitation with 10% trichloroacetic acid were assayed for radioactivity and the results were corrected for radioactivity found in identically treated control mixtures in which the serum sample had been replaced with 0.15 M NaCl.

Preparation, Purification, and Labeling of Antibodies and Antigens

Preparation of Hemoglobin Chain Antigens—Globin obtained from unfractionated hemolysates of hepatoch-trated washed red cells from adult New Zealand White rabbits was used for separation of hemoglobin α and β chains via CM-cellulose chromatography at pH 2.7 (12, 13). Isolated chains, when freed of solvents, were either re-chromatographed several times or stored as lyophilized powder.

Preparation of Antisera—Antisera against individual hemoglobin chains, rabbit IgG, and human IgG were developed in outcrossed goats; antisera to goat IgG were prepared in rabbits. In a typical immunization schedule, ~10 mg of protein, thoroughly emulsified (14) with an equal volume of Freund's complete adjuvant (Difco), were given subcutaneously, followed at 28, 35, and 42 days by 10 to 15 mg of protein without adjuvant.

Preparation of Antisemorphs—Antisemorphs were prepared from ribosome-free, 140,000 X g supernatants was stored at -85° for 184 days; 36%, 152 days; 9%, 138 days; 9%, 93 days; and 0% after 24, 10, and 9 days.

4-Amino Acid Incorporation into Completed Hemo- globin and Nascent Polysome-bound Chains—With the use of procedures detailed elsewhere (10, 20), 1 ml of packed rabbit reticulocytes was incubated with either 2 mCi (24 Ci per mmole) of L-[aH]lysine or a mixture of 2.5 mCi (74 Ci per mmole) of L-[3H]lysine and 50 &i (252 mCi per mmole) of L-[14C]lysine. Hemoglobin uniformly labeled with L-[14C]lysine (50 μCi; 312 μCi per μmole) was prepared from ile-6 hour incubations of 0.5 ml of reticulocytes from the same subjects from which samples for the synthesis of globin were obtained. Final concentrations of all amino acids (including the one labeled) approximated the values given by Phillips (21). Polysomes were harvested and stored as described above. Hemoglobin recovered from ribosome-free, 140,000 X g supernatants was stored at -85°.

In order to forestall later nonimmunological trapping of trace quantities of iodinated antibody. Without such pretreatment, as much as 50% of total radioactivity was trapped by antigen-free Sepharose. After pretreatment, 125I- or 3H-labeled antibodies were added, and final volumes of 0.7 to 1.1 ml were allowed to re-act for 1 hour. Centrifuged Sepharose-free supernatants were recovered and assayed for radioactivity. In each set of assays, one control tube contained antigen-free Sepharose and in another Sepharose was replaced with 0.15 M NaCl solution. Soluble radioactivity remaining in the two kinds of controls was approximately the same. Estimation of the proportion of radioactive antibody bound depended on comparison with the 0.15 M NaCl control, viz., (control supernatant cpm) — (sample supernatant cpm) / (control supernatant cpm) = % bound.

Preparation and Sedimentation of Polysomes

Isolation of Reticulocyte Polysomes—Blood containing 80 to 90% reticulocytes was collected in heparin from hearts or ear veins of adult rabbits made anemic by injection of phenylhydrazine (10). Washed cell slurries were osmotically disrupted with 4 volumes of chilled 2 m/m MCl and, after 45,5 hours, were rested with 1 vol-

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Density Gradient Sedimentation of Polysomes—Polysomes (0.2 to 1.6 ml containing 4 to 16 A260 units per ml) were layered over 0.44 to 0.88 m sucrose gradients in TKM buffer and centrifuged (at 4°) either at 22,000 rpm through 28-m gradients for 5 hours in

\[ \text{Density Gradient Sedimentation of Polysomes—Polysomes (0.2} \]

1 The abbreviation used is: IgG, immunoglobulin G.
of 0.2 mg of trypsin was added after 1.5 hours and the incubations were continued for a total of 4 hours. The centrifuged pH 8.2 supernatant and one wash of precipitate from each trypptic digest were pooled, resuspended in water, polyethylated to remove residual NH₄HCO₃, and resuspended in 0.026 M formic acid. Clear solution from each digest, containing approximately two-thirds of the I'H and I'C radioactivity present at the start of digestion, was applied to a column (1.9 x 230 cm) of Sephadex G-25 containing 0.026 M formic acid (24). The effluent peptides from each column, recognized by A₂₆₀ monitoring, were pooled (according to size) into four groups containing (in order of group elution): lysine-bearing tryptic peptides (24) a₁, a₁₀, a₆, a₈ + b₁, b₂, b₂, b₆, b₆, and b₁₀ in the first pool; a₂, a₃, a₃, a₉, a₁₁, a₁₀, and a₁₃ in the second; a₂, a₃, a₃, b₁, b₆, b₆, and b₁₀a in the third and a₂ in the last. After lyophilization, each pool was fractionated by preparative pH 4.7 paper electrophoresis and subsequent paper chromatography in Hill's solvent (24). Peptides were localized on paper by use of guide margins developed with 0.2% ninhydrin in acetone. Following paper chromatography, unstained strips were eluted overnight with 0.13 M formic acid. Lyophilized peptides were resuspended in the same solvent; thereafter approximately one-fifth of each resuspension was analyzed for amino acids in order to establish identity and purity of peptide; the remainder of each was analyzed for I'H and I'C disintegrations per min.

Assumptions Used in Calculation (5)—The following equivalents were used: (a) molecular weight of ribosomes = 4 X 10⁶; 1 A₂₆₀ unit of ribosomes = 80 µg = 22.5 pmole; (b) molecular weight of IgG = 1.5 x 10⁶; 1 µg of IgG = 6.7 pmole; (c) molecular weight of globin chain = 1.6 x 10⁴; 1 µg of globin chain = 62 pmole.

RESULTS

Purification and Immunological Assay of Antibodies—RNase activity and attendant polysome breakdown were detectable whenever ~20 µl or more of serum were mixed with 1 ml of polysomes containing 10 A₂₆₀ units. Removal of RNase from antisera was accomplished via immunoadsorption. The extent of purification of desorbed antibodies was assessed through comparison of quantities of protein desorbed from antigen-bearing Sepharose versus quantities desorbed from antigen-free Sepharose (Table 1). Purification was usually several hundred-fold, and it is not surprising that all antibodies isolated by immunoadsorption were free of detectable RNase.

In Table 1, recoveries of purified antibodies against hemoglobin α chains are approximately comparable to recoveries of various purified anti-IgG. The exceptionally low yields associated with purified goat anti-rabbit hemoglobin β (Table 1) were earlier forecast by two findings: namely, (a) rabbit β chain provoked precipitating antibodies in only two of 12 immunized goats, in contrast, for example, to five responders of five goats immunized with rabbit α; and (b), when detectable in whole sera, precipitating antibodies against rabbit β were evanescent during the course of serial bleedings. Following immunoadsorptive purification, anti-rabbit hemoglobin β preparations became totally unreactive on Ouchterlony plates, even when assayed at antibody concentrations of 1 mg per ml and, in this respect, were quite unlike anti-rabbit α. Purified anti-rabbit β preparations from six of 12 goats immunized did, however, contain nonprecipitating antibodies. These were demonstrated by i₁H-labeled anti-β binding (Fig. 1) to Sepharose-linked β under conditions in which nonimmunological binding of trace quantities of protein was precluded by prior saturation of Sepharose with normal goat serum.

Specificity of Antibodies against Hemoglobin Chains—Although the precipitating reactions found for anti-rabbit hemoglobin α chain on Ouchterlony plates were immunologically specific (i.e. anti-α did not react with β chain), the possible existence of cross-reactive nonprecipitating antibodies was not excluded. Exclusion was essential because our later studies depended on complete immunological specificity. Specificities of both anti-α and anti-β sera were confirmed by immunoadsorption, as described above.

Preparation and Isolation of Hemoglobin Tryptic Peptides from Polysomes

The non-normalized y intercepts of radioactive nascent peptide patterns, i.e. Dintzis-Naughton plots (12, 13), obtained from untreated polysomes were used as a measure (23) of the relative numbers of α and β chain-synthesizing ribosomes. For this purpose we prepared mixtures of ~0.1 µCi of [I]lysine-labeled polysomes, 0.7 µmol of carrier globin, and ~0.1 µCi of globin uniformly labeled with the use of tritiated glycerol. In each case, two (i.e. anti-α) and three (i.e. anti-β) digestion mixtures were used: (a) molecular weight of ribosomes = 4 x 10⁶, 1 A₂₆₀ unit of ribosomes = 80 µg = 22.5 pmole; (b) molecular weight of IgG = 1.5 x 10⁶, 1 µg of IgG = 6.7 pmole; (c) molecular weight of globin chain = 1.6 x 10⁴, 1 µg of globin chain = 62 pmole.

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TABLE I
Recovery and purity of antibodies following immunoadsorption

For all entries, molar ratio between desorbed antibody and antigen linked to Sepharose was < 0.25 for anti-IgG and < 0.07 for antihemoglobin chains. Under these conditions, incomplete binding of applied specific antibody is minimized. Specific to nonimmunological ratios are measures of purity; "specific" denotes weight of antibody protein desorbed from appropriate antigen linked to Sepharose, and "nonimmunological" refers to weight of protein bound to the same quantity of antigen-free Sepharose. Estimates of nonimmunologically bound protein depend on (a) volume of packed Sepharose used for "specific" adsorption and (b) capacity of 1 ml of packed Sepharose 2B to adsorb, regardless of serum load, an average of 12 mg of protein from serum of a nonimmunized animal. Thus, for example, in the second row 1.95 mg of anti-β were recovered from 33 ml of Goat 388 serum (0.06 mg per ml) following desorption from 1 ml of packed β chain-bearing Sepharose; 0.012 mg of serum protein is nonimmunologically bound by this quantity of Sepharose and therefore the specific adsorption to nonimmunological adsorption ratio is 1.95:0.12 = 16.5. All recoveries from antisera to hemoglobin chains were obtained after preliminary exposure to Sepharose bearing the contrary hemoglobin chain, e.g., anti-α was first exposed one to three times to β chain-bearing Sepharose. Total quantities of particular antibodies initially present in antisera are only approximate, since recoveries are not corrected for small remnants of unbound antibody and losses of immune complexes during Sepharose washing and transfer.

Table:<br>

| Antiserum                  | Immunized animal no. | Recovery | Specific adsorption + nonimmunological adsorption |
|---------------------------|----------------------|----------|-----------------------------------------------|
| Goat anti-rabbit hemoglobin β | 591                  | 85       | 170β                                           |
| Goat anti-rabbit hemoglobin α | 588                  | 60       | 165                                             |
| Goat anti-rabbit IgG        | 590                  | 304      | 52β                                            |
| Goat anti-human IgG         | 594                  | 238      | 480                                             |
| Rabbit anti-goat IgG        | Pool                 | 244      | 204                                             |

* Micrograms of antibody protein desorbed per ml of antiserum applied.<br>† Mean of two separate preparations.

and anti-β were therefore further tested by binding iodinated antibodies to excess antigen coupled to Sepharose. Testing was performed, once again, under circumstances in which nonimmunological binding was prevented. We were surprised to find that binding patterns of purified iodinated anti-rabbit α and anti-rabbit β were marred by considerable immunological nonspecificity. For example, when assayed in the presence of a 100 μ excess chain antigen, 18 to 23% of 125I-labeled anti-α, despite extensive prior exposure to Sepharose-β chain during purification, is bound by β chain-bearing Sepharose (cf. 27% to α chain-bearing Sepharose), even when the β chains come from a succession of chromatographic fractions increasingly remote from α. Such immunological nonspecificity could be explained either by residual cross-reacting antibody in purified preparations or by cross-contamination of α and β antigens with one another. The latter possibility was tested by repeated isolation and re-chromatography on CM-cellulose of individual hemoglobin chains. The section of chain peak re-chromatographed in each cycle was varied in an attempt to remove trace cross-contamination of α and β chains with one another. Following five such cycles, the now highly purified rabbit hemoglobin α and β chains were coupled to Sepharose and tested in antigen excess for their ability to bind each of several lots of iodinated anti-rabbit α and anti-β. The original lots of iodinated antibodies, which had been immunologically nonspecific when assayed against hemoglobin chains isolated once, were entirely specific when tested (Table II) against Sepharose linked to repeatedly chromatographed chains. Clearly, cross-contamination of antigens rather than residual unwanted antibodies was the source of prior nonspecific-

FIG. 1. Binding of mixtures of unlabeled and 125I-labeled purified goat anti-rabbit hemoglobin β chain to constant amounts (128 pmoles) of rabbit β covalently linked to 20 μl aliquots of packed Sepharose. Derivation of abscissa and right ordinate values depend on prior estimates of the fraction of antibody protein remaining immunologically active after purification (~90% in this case) and still later after iodination (45% in this case); estimates were obtained at immunoglobulin to antigen ratios substantially less than those plotted. Immunological activity in purified but noniodinated immunoglobulin was deduced from comparisons between binding found for (a) labeled antibody which had been diluted with unlabeled antibody and (b) equivalent masses of undiluted labeled antibody; because of imprecision in measuring small amounts of protein, estimates of activity in unlabeled protein (which depend on several quantitative determinations) are necessarily approximate. ●—●, moles of anti-rabbit β-Sepharose; ○—○, percentage of active antibody bound/antibody applied.

TABLE II
Binding of purified 125I-labeled goat anti-rabbit hemoglobin chain to highly purified rabbit hemoglobin chains linked to Sepharose

Hemoglobin α and β chains were re-chromatographed four or five times prior to covalent coupling to Sepharose. Highly purified hemoglobin β (679 pmoles), linked to Sepharose, was used for each binding assay. The percentage of 125I-labeled antibody bound, of the 5 to 20 pmoles applied, is expressed relative to that found in control incubations lacking Sepharose. Antibody binding to antigen-free Sepharose (not shown) was less than 6% in all instances.

Table:<br>

| Antibody (Lot) | Hemoglobin α | Hemoglobin β | % antibody bound |
|----------------|--------------|--------------|-----------------|
| Anti-α (382B) | 21           | 5            |
| Anti-α (382B) | 21           | 1            |
| Anti-β (591D) | 0            | 34           |
| Anti-β (591C) | 0.6          | 45           |
| Anti-β (591D) | 0            | 28           |
FIG. 2. Binding of \(^{131}1\)-labeled anti-rabbit hemoglobin \(\beta\) (5.5 pmoles of active antibody protein, 26 nCi per pmole) from Goat 591 (\(\bullet\)) and, separately, \(^{125}1\)-labeled anti-rabbit IgG (9.4 pmoles of active antibody protein, 15 nCi per pmole) from Goat 596 (\(\bigcirc\)) to rabbit reticulocyte polysomes (~100 pmoles of ribosomes as polysomes in each instance). Antibody-polysome mixtures (0.2 ml) were sedimented through 28-ml sucrose gradients and fractionated directly into scintillation vials. Counts from uppermost fractions (on right) are omitted.

ity (e.g., later discussion of a chromatographically aberrant \(\alpha\) chain). The point is an important one because the use of small quantities of highly purified individual chains as final test antigens enables us to show not only that each of the antibodies later used for reactions with reticulocyte polysomes is entirely specific for a particular hemoglobin chain but also that hemoglobin chains chromatographed once, even though contaminated with \(~1\)% of unwanted chain, are satisfactory for both immunization and immunoabsorption. The example is not unique; a similar situation occurred with immunoabsorptively purified antibodies against mouse \(\alpha\) and \(\beta\) chains as well as against human \(\alpha\), \(\beta\), and \(\gamma\) chains, when in each case, immunological nonspecificity was seen with hemoglobin chains chromatographed once but vanished when highly purified, re-chromatographed chains were used as antigens.

**Binding of Iodinated Antibodies to Polysomes**—The differential binding of purified \(^{131}1\)-labeled anti-rabbit \(\beta\) and \(^{125}1\)-labeled anti-rabbit IgG to rabbit reticulocyte polysomes sedimented in individual sucrose density gradients is shown in Fig. 2. In this instance, 62% of the immunologically active anti \(\beta\) applied was bound and resulted in a recognizable polysome profile, whereas only 2.6% of applied anti-IgG appeared in the polysome region. From this and related analyses, including many sedimentation assays of polysomes allowed to react with iodinated anti-rabbit \(\alpha\) (e.g. in Fig. 3), it is evident that antibodies developed against completed hemoglobin chains can react with nascent hemoglobin chains. In contrast, nonimmunological associations between reticulocyte polysomes and purified antibodies to antigens presumably absent, typified by \(^{131}1\)-labeled anti-IgG binding in Fig. 2, are negligible and usually involve <1% of applied antibody.

The capacity of reticulocyte polysomes to bind rabbit anti-hemoglobin \(\beta\) chain, as seen in Fig. 4, can be compared with the binding capacity of \(\beta\) chain-bearing Sepharose (seen in Fig. 1)

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1 S. H. Boyer, unpublished observations.
Fig. 4. Binding of varying quantities of $^{125}$I-labeled Goat 591 anti-rabbit hemoglobin $\beta$ chain to reticulocyte polysomes. $\bullet$, $0$; $0$, $41$ pmoles of ribosomes as polysomes were used; $\square$, $41$ pmoles; $\blacksquare$, $78$ pmoles. All polysomes came from the same batch used for analysis depicted in Fig. 3. Each point was obtained after sedimentation of an antibody-polysome mixture through $4.4$ ml containing discontinuous concentrations of sucrose. Two different lots of $^{125}$I-labeled anti-$\beta$ were used. Data shown as $\bullet$ and $\bigcirc$ were obtained with the $^{125}$I-labeled anti-$\beta$ lot (48% maximal immunological activity after iodination) used in Fig. 1 for measurement of antibody binding to $\beta$ linked to Sepharose. This lot was used here in varying amounts at constant specific activity ($24$ nCi per pmol). A different lot of labeled antibody (34% maximal immunological activity after iodination) was applied at constant specific activity ($30$ nCi per pmol) for $\square$ point assays, whereas for $\blacksquare$ point assays it was diluted with unlabeled Goat 591 anti-$\beta$ from the same batch of purified antibody ($\sim90\%$ maximal immunological activity before iodination). $\square$ points are not connected because the estimate of $90\%$ maximal immunological activity in unlabeled anti-$\beta$ (on which point locations depend) is only approximate (q.v. legend to Fig. 1). The percentage of active antibody bound/applied (right ordinate) is shown only for $\bullet$ and $\bigcirc$; a similar plot can be constructed for $\square$ and $\blacksquare$.

for the same lot of iodinated antibody. For any particular load, the moles of anti-$\beta$ bound per mole of polysomal ribosomes represent slightly more than one-half of the quantity bound per mole of $\beta$ chain linked to Sepharose. This result is unexpected, for it suggests that notably all of the polysomes engaged in $\beta$ synthesis (approximately one-half of all polysomes; see below) bear nascent chains which can bind antibody raised against completed $\beta$ chains. Alternatively, it is possible that a few nearly completed $\beta$ chains can each bind 2 or more antibody molecules. A more likely explanation is that the binding capacity of $\beta$ chain-bearing Sepharose has been somewhat underestimated, because not all of $\beta$ measured as a protein remains active as an antigen. Since the portion which is antigenically inactive cannot be directly assessed, the actual fraction of $\beta$ nascent chains which can bind anti-$\beta$ is uncertain. Flats like Fig. 4 nonetheless retain their principal value, which is to serve as a gauge for estimating optimal quantities of antibody later required for immunoprecipitation of polysomes.

Reactions between anti-chains and polysomes are also useful in assessing an aspect of immunological specificity which has until now been ignored, viz. the possibility that purified antibody preparations might react with nonhemoglobin antigens. In general, the hemoglobin chains used for both immunization and later immunoadsorption were chromatographed only once. Trace quantities of nonhemoglobin antigens were undoubtedly present. Antibodies produced against these might be recovered during adsorption-desorption from Sepharose bearing contaminated antigen. That such unwanted antibodies are absent from purified preparations can be shown by comparisons of the binding of antibody to Sepharose-linked antigen and the binding of the same antibody to polysomes. For example, under conditions in which molar ratios of antigen to antibody are $>100:1$, $34\%$ of the radioactivity in iodinated anti-$\beta$ preparation 591B (Table II) was bound to purified $\beta$ chain linked to Sepharose and $34\%$ to polysomes. Similar equivalence was seen, at different percentages, with other iodinated anti-$\beta$ preparations. We interpret these findings as inconsistent with an appreciable contamination of anti-$\beta$ with antibodies to nonhemoglobin antigens. Our interpretation rests on the obvious differences between the kinds and concentrations of nonhemoglobin proteins present in $\beta$ chain preparations isolated from the blood of nonanemic rabbits and those present in reticulocyte polysomes. Because of such differences in antigenic profiles, any antibody developed against nonhemoglobin antigens should bind more or less well to polysomes than they do to isolated hemoglobin $\beta$ chain. Yet, even when anti-$\beta$ preparations are tested against gross excesses of antigen, so that non-$\beta$ antigens might have every chance for expression, there is no difference between the quantity of antibody bound to polysomes and that bound to chain. Accordingly, these anti-$\beta$ preparations are free of unwanted antibody. Similar analytical precision was not possible for $^{131}$I-labeled anti-$\alpha$ reactions in which polysome-bound anti-$\alpha$ is incompletely resolved from the tailing of unbound anti-$\alpha$ (q.v. below) during density gradient sedimentation. Nonetheless, under conditions of gross antigen excess, the percentage of a given lot of iodinated anti-$\alpha$ bound to Sepharose-$\alpha$ and the percentage bound to nascent $\alpha$ chains is approximately comparable. This, once again, suggests that antibodies to nonhemoglobin proteins are absent.

**Contrasting Sizes of $\alpha$ and $\beta$ Chain-synthesizing Polysomes—** Differences in rabbit (Animal 90) reticulocyte polysome profiles formed by binding $^{125}$I-labeled anti-$\alpha$ and $^{131}$I-labeled anti-$\beta$ are evident in Fig. 3. Iodinated anti-$\alpha$, on the average, binds to considerably smaller polysomes than does anti-$\beta$. In contrast, the A$_{260}$ patterns are identical for each analysis and are indistinguishable from that given by antibody-free polysomes; degradation by RNase is thus excluded as an explanation for the differences between the two iodinated antibody-labeled polysome profiles. Although precise estimates of the mean sizes of anti-$\alpha$-labeled polysomes and anti-$\beta$-labeled polysomes are obtained by the tailing of unbound $^{125}$I-labeled antibodies into the region of polysomes containing 1 and 2 ribosomes per mRNA, approximations are still possible. By inspection, the mean size of polysomes (Fig. 3) labeled by $^{131}$I-anti-$\beta$ is somewhat greater than 6 ribosomes per mRNA and, in any case, slightly larger than the mean of untreated polysomes ($\sim5$ ribosomes per mRNA) and substantially larger than the mean size of polysomes labeled with $^{131}$I-anti-$\alpha$ (about 2 ribosomes per mRNA). Consequently, in this instance, the $\beta:\alpha$ ratio for mean polysome sizes is $>6:4$.

In Fig. 5, a similar $\beta:\alpha$ ratio for mean polysome sizes was deduced in a second rabbit (Rabbit 89) from (a) the $\alpha$ polysome A$_{260}$ profile (mean size, $\sim4$ ribosomes per mRNA) remaining after immunoprecipitation of the bulk of $\beta$ polysomes by successive use of goat anti-rabbit $\beta$ and rabbit anti-goat IgG, and (b) the $\beta$-polysome A$_{260}$ profile (mean size, $\sim6$ ribosomes per mRNA) left after immunoprecipitation of most $\alpha$ polysomes through serotonin use of goat anti-rabbit $\alpha$ and rabbit anti-goat...
and manipulations just described, can be converted to \(^{3}H\)-labeled \(\alpha\) and \(\beta\) polysome profiles. Although Hunt et al.'s method (5) is subject to errors and uncertainties in each variable (e.g. allelic variations between individuals in isoleucine (20) and valine (26, 27) residues present at particular positions) and furthermore is potentially affected by trace contributions from isoleucine-rich nonhemoglobin nascent chains, it is nonetheless grossly valid, as evident from comparisons of Fig. 6 with Figs. 3 and 5. Among untreated polysomes in the top panel of Fig. 6, the derivative \(\alpha\) polysome mean size is \(~3.5\) ribosomes per mRNA, whereas \(\beta\) mean size is \(~6.5\) ribosomes per mRNA. Following removal of the majority of \(\alpha\) polysomes through use of anti-\(\alpha\) and anti-antibody (center panel, Fig. 6), \(\beta\) mean polysome size by both \(A_{380}\) and \(^{3}H\) radioactivity remains \(~6.5\) ribosomes per mRNA. In the bottom panel of Fig. 6, the mean size of \(\alpha\) polysomes remaining after immunological removal of \(\beta\) polysomes is \(~4\) ribosomes per mRNA, whether judged by \(A_{380}\) or \(^{3}H\) radioactivity. Thus in the control (top) panel of Fig. 6, \(\beta:\alpha\) mean polysome size ratio is \(~6.5:3.5 = 1.86\), whereas after immunoprecipitation (center and bottom panels) it is \(~6.5:4 = 1.62\), as measured by two different criteria (\(A_{380}\) and radioactivity).

Relative Numbers of \(\alpha\) and \(\beta\) Chain-synthesizing Ribosomes—

The foregoing estimates of mean \(\alpha\) and \(\beta\) polysome size are later used, along with estimates of the relative numbers of ribosomes, to calculate the numbers of active \(\alpha\)- and \(\beta\)-mRNA. Relative numbers of \(\alpha\) and \(\beta\) chain-synthesizing ribosomes were deduced from Dintzis-Naughton plots (12, 13) of \([^{3}H]labeled nascent peptides (Fig. 7). In each section of Fig. 7, \(y\) axis intercepts, produced by regression slopes of relative specific activity along ordinates versus chain sequence position (12, 13) of peptide lysine residues along \(abscissa\), represent the hypothetical sum of peptide lysine residues and thus the sum of nascent chains, i.e. ribosome-associated chains. Since each synthetically active ribosome is associated with only one nascent chain, the \(y\) intercept of each \(\alpha\) and \(\beta\) regression line is the sum of ribosomes actively involved in the synthesis of that chain. The ratio of \(y\) intercepts (where \(x = 1\)) is a measure (5) of the relative numbers of ribosomes engaged in \(\alpha\) and \(\beta\) chain synthesis. However, before calculating these numbers, \(y\) intercept ratios (which depend on \(^{3}H\) contributions from nascent peptides and \(^{14}C\) contributions from uniformly labeled hemoglobin of the same rabbit) must be corrected for any \(\alpha\) versus \(\beta\) imbalance in the radioactivity of the batch of uniformly labeled \(^{14}C\) chains used as an index of recovery. Such calculations and corrections can be illustrated by reference to the Dintzis-Naughton plots shown in Fig. 7. The \(\beta:\alpha\) ratio of \(y\) intercepts for Rabbit 90 (upper panel) is 1.015. This ratio, when corrected for a \(\beta:\alpha\) ratio of 1.133 in the uniformly labeled \(^{14}C\) chains used, gives a \(\beta:\alpha\) ratio of 1.17 for the relative numbers of synthetically active ribosomes. In the lower panel of Fig. 7, the \(\beta:\alpha\) ratio of \(y\) intercepts for Rabbit 109 is 0.87, which, when corrected for a \(\beta:\alpha\) ratio of 1.19 in \(^{14}C\)-labeled chains, yields a \(\beta:\alpha\) ratio of 1.04 for synthetically active ribosomes.

In deriving the preceding estimates of relative numbers of \(\alpha\) and \(\beta\) chain-synthesizing ribosomes, we have assumed that the completed \(\beta\) chains, shown as stippled areas in Fig. 7, are covalently bonded to ribosomes. The existence of relatively constant proportions (\(~10\%) of completed \(\beta\) chains and an attendant lack of significant amounts of completed \(\alpha\) chains are universal, as inferred from the location of \(x\) intercepts in every one of eight Dintzis-Naughton plots. These include two plots obtained after preparative sucrose density gradient partition of previously pelleted and rehydrated polysomes. Although the
latter results effectively preclude contamination of nascent peptides with completed chains from hemoglobin tetramers (where both α and β chains are found to be equally well labeled), we cannot at this time exclude the possibility that ribosome-bound, completed β chains have been secondarily attached. If completed β chains are secondarily attached, it follows that they are probably attached to ribosomes which are also synthesizing a nascent chain and must therefore be excluded from calculations of synthetically active ribosomes based on y intercepts. In later calculation of relative numbers of synthetically active α- and β-mRNA (Table III), we shall use both kinds of estimates of β:α ribosome ratio, i.e. with and without the contribution of completed β chains.

DISCUSSION

Mean Sizes of β Chain-synthesizing Polysomes versus α Chain-synthesizing Polysomes—Each of the approaches used for comparing the mean number of ribosomes attached to β-mRNA, i.e. mean β polysome size, with mean α polysome size is subject to one or another kind of bias. In general, such biases are in the direction of producing underestimates in the β:α ratio for mean sizes. For example, β:α estimates of >6:4 (Fig. 3), which depend on sedimentation of polysomes previously coated with iodinated antibodies to hemoglobin chains, are prejudiced by differences in the target polysomes. Because α polysomes are on the average smaller than β polysomes, it is expected that the proportion of α polysomes in the single ribosome per mRNA category will be greater than the proportion of β polysomes in this class. Consequently, the proportion of single-ribosome polysomes which bear a nascent chain too short to react with antibodies developed against whole chains will be relatively greater among α polysomes than among β polysomes. To a lesser extent, the same will be true for polysomes bearing 2 or 3 ribosomes per mRNA. The resulting skewed distribution of unlabeled ribosomes leads to overestimation of the mean size of α polysomes and corresponding underestimation of the β:α ratio for mean sizes of polysomes.

The ratio β:α = 6:4 between mean numbers of ribosomes per mRNA, adduced by sucrose gradient sedimentation (Fig. 5) of what remains after polysome precipitation using anti-α (i.e. β polysomes) and what remains after precipitation using anti-β (i.e. α polysomes), is also an underestimate. In this case, residual α polysomes are undoubtedly left in the anti-α postprecipitation supernatant, whereas residual β polysomes are left in the anti-β supernatant.

The sources of potential underestimation of the β:α ratio for mean polysome sizes, intrinsic to the methods used in Figs. 3 and 5, do not influence estimates (Fig. 6) based on differential α and
Fig. 7. Dintzis-Naughton plots (12, 13) of relative $^{3}$H:I:4C specific activities (ordinates) versus chain sequence positions (abscissae) found in nascent chain tryptic peptides from pelleted, rinsed reticulocyte polysomes of Rabbit 90 (upper panel) and Rabbit 109 (lower panel). Regression slopes are fitted by least squares; points from NH$_{2}$-terminal peptides $\alpha_1$ or $\alpha_1 + \alpha_2$, or both, are shown but, because of their known heterogeneity, are omitted in regression line calculation. Points shown in duplicate were obtained from separate Sephadex G-25 fraction pools during purification, and the mean of such duplicates was used in line fitting. Stippled areas at bottom of each panel are subtended by verticals drawn to $\beta$ chain regression line from the COOH terminus (position 146) of the $\beta$ chain (1) and represent statistically significant quantities (p < 0.05) of uniformly labeled $\beta$ chain. Solid areas in each panel denote uniformly labeled $\alpha$ chains. The $x$ axis intercepts of $\alpha$ chains are not significantly different (p > 0.1) from the known $\alpha$ COOH terminus; therefore, correction for ribosomes bearing completed $\alpha$ chains does not figure in calculations in text and Table III. O---O, $\alpha$ chain peptides; ●---●, $\beta$ chain peptides.

Nonetheless, have their own kind of flaw. For example, the calculated partition of $\alpha$ and $\beta$ polysomes in each fraction of Fig. 6 depends on prior measurement of $^{3}$H:I:4C ratios in uniformly labeled $\alpha$ and $\beta$ chains obtained from whole hemoglobin molecules (5). These latter measurements were invariably marred (in each of seven rabbits) by an aberrant $\alpha$ chain which, during CM-cellulose chromatography at pH 2.7, lies under the peak and descending limb of the $\beta$ chain. Although this aberrant $\alpha$ chain represents only $\sim 1%$ of all $\alpha$ chain globin, it is nonetheless richer in isoleucine residues and poorer in valine residues than $\beta$ chain per se and thus leads to a spurious elevation of the $[3H]$/isoleucine to $[4C]$valine ratio under the nominal $\beta$-peak. The effect of this alteration on the calculated partition of counts between $\alpha$ and $\beta$ polysomes has undoubtedly contributed to uplifting the $\beta$-plateau underlying the otherwise isolated $\alpha$ polysome peak in the lower panel of Fig. 6. While such effects are probably too small to have much influence on the positions of $\alpha$ and $\beta$ polysome peaks as such, they nevertheless can influence the shape of the polysome profiles. This distortion will be especially manifest in areas where polysomes for the two kinds of chain overlap, e.g., in the 3 to 5 ribosomes per mRNA region. By contrast, distortion should be negligible in those regions which are largely occupied by $\beta$ polysomes, i.e., the >6 ribosomes per mRNA region. The resultant skewing of $\beta$ polysome profiles to the lighter side may lead to mean sizes of $\beta$ polysomes slightly smaller than actually present and, to this degree, result in a modest underestimation of the difference between the average sizes of $\beta$ and $\alpha$ polysomes.

Despite the uncertainties which beset each method (Figs. 3, 5, and 6) for estimating mean polysome size, the resulting $\beta$:$\alpha$ ratios for mean sizes are remarkably similar ($\sim 6.4$ to $\sim 6.5$:3.5, i.e., 1.5 to 1.86) and, even without the real possibility of ratio underestimation, somewhat greater than the previously reported (6, 7) ratios of 1.3 to 1.4. In any case, we corroborate the mean size differences between $\alpha$ and $\beta$ polysomes which heretofore had been known (5-7) only by the indirect and partially flawed method of differential labeling of isoleucine and valine residues. The immunological methods exemplified in Figs. 3 and 5 should be especially useful for studies in those species in which isoleucine residues are absent.

Relative Numbers of Synthetically Active $\alpha$- and $\beta$-mRNA---The two kinds of $\beta$:$\alpha$ ratios, i.e., ratios for (a) relative numbers of ribosomes engaged in synthesis of each chain (Fig. 7) and (b) mean numbers of ribosomes attached to each kind of mRNA (Figs. 3, 5, and 6), are used in Table III to calculate the relative numbers of $\alpha$- and $\beta$-mRNA active in each of several reticulocyte preparations. In Table III relative numbers of $\alpha$- and $\beta$-mRNA are, for example, estimated for Rabbit 90 by substituting the relative numbers of $\alpha$ versus $\beta$ ribosomes (1.17 versus 1, upper panel in Fig. 7) and mean polysome sizes (4 ribosomes per $\alpha$-mRNA and 6 ribosomes per $\beta$-mRNA, Fig. 3) in the expression

$$\text{No. active } \alpha\text{-mRNA } = \frac{\text{relative No. ribosomes for } \alpha\text{ chains}}{\text{mean No. ribosomes per } \alpha\text{-mRNA}}$$

$$\text{No. active } \beta\text{-mRNA } = \frac{\text{relative No. ribosomes for } \beta\text{ chains}}{\text{mean No. ribosomes per } \beta\text{-mRNA}}$$

viz. $(1.17/4)/(1.17/6) = 1.28.$ In making this estimate we suppose that completed $\beta$ chains (stippled areas in Fig. 7) are covalently bonded to polysomes rather than, as discussed earlier, secondarily bound. If completed $\beta$ chains are assumed to be secondarily attached, the number of ribosomes active in $\beta$ synthesis is reduced from 1.17 to 1.08, and the $\alpha$:$\beta$ ratio for calculated numbers of active mRNA is increased to 1.39. Similar calculations are
Calculations of relative numbers of active hemoglobin \( \alpha \)- and \( \beta \)-mRNA

Calculations are based on the expression (No. active \( \alpha \)-mRNA)/(No. active \( \beta \)-mRNA) - (relative No. ribosomes bearing \( \alpha \) chains/mean No. ribosomes per \( \alpha \)-mRNA)/(relative No. ribosomes bearing \( \beta \) chains/mean No. ribosomes per \( \beta \)-mRNA). Relative numbers of synthetically active ribosomes for each rabbit are derived from \( y \) intercepts at \( x = 1 \) in Fig. 7 and include contributions of completed \( \beta \) chains. In parentheses, contributions of completed \( \beta \) chains attached to ribosomes are omitted in derivation of relative numbers of synthetically active ribosomes and in calculation of relative numbers of mRNA. Mean polysome sizes for Rabbit 90 are based on \(^{141} \)I profiles in Fig. 3 and for Rabbit 109 on differential labeling of isoleucine and valine in Fig. 6.

| Rabbit no. | Relative No. of synthetically active ribosomes | Mean No. of ribosomes per mRNA | Calculated relation No. of mRNA |
|-----------|---------------------------------------------|-------------------------------|-------------------------------|
| 90        | \( \alpha \) 1.17 (0.98) \( \beta \) 4 6 | 1.28 (1.39) \( \alpha \) 1 \( \beta \) 1 |                          |
| 109       | \( \alpha \) 1.04 (0.93) \( \beta \) 4* 6.5* | 1.56 (1.74) \( \alpha \) 1 \( \beta \) 1 |                          |
| 109       | \( \alpha \) 1.04 (0.93) \( \beta \) 3.5* 6.5* | 1.78 (2.04) \( \alpha \) 1 \( \beta \) 1 |                          |

* Mean polysome sizes based on top panel of Fig. 6.

\( \beta \) Mean polysome sizes derived from composite of center and bottom panels of Fig. 6.

Also shown (Table III) for Rabbit 109, for which the \( \alpha/\beta \) ratio for calculated numbers of mRNA ranges, depending upon assumptions, between 1.56 and 2.04. Since the relative excess of \( \alpha \)-mRNA will increase according to the degree to which the difference between \( \alpha \) and \( \beta \) polysome mean size has been underestimated, we believe that the actual excess is somewhat greater than that given in Table III and larger than the 1.3- to 1.4-fold excess indirectly adduced by Lodish (6) and Lodish and Jacobsen (7).

Estimates in Table III apply only to active mRNA, i.e. mRNA actually being translated on soluble polysomes. Whether there is an equivalent excess of \( \alpha \)-mRNA among inactive templates is uncertain. The only straw in the wind is the finding of translatable rabbit \( \alpha \)-mRNA in supernatants remaining after reticulocyte ribosomes have been pelleted (28); little translatable \( \beta \)-mRNA is evident in this fraction. Such nonpolysomal \( \alpha \)-mRNA represents \(~25\%\) of all detectable \( \alpha \) chain template (28). This contribution, when added to the roughly 1.5-fold excess of \( \alpha \)-mRNA found for a composite of values in Table III, suggests that the actual proportion of \( \alpha \)-mRNA, in all its forms, may be approximately twice that of \( \beta \)-mRNA. We have used this presumptive 2-fold \( \alpha \) excess as part of a hypothesis (8) for the ways in which differential restriction to initiation of \( \alpha \)- and \( \beta \)-mRNA translation (6, 7), mRNA-dependent differences in polysome size, unequal numbers of kinds of mRNA, and ubiquitous but always evolutionarily recent \( \alpha \)-gene duplication in mammals may be interrelated.

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