*POLYRIBOSOMAL SYNTHESIS AND ASSEMBLY OF THE H AND L CHAINS OF GAMMA GLOBULIN**

By A. L. Shapiro,† M. D. Scharff,† J. V. Maizel, Jr.,† and J. W. Uhr

DEPARTMENTS OF CELL BIOLOGY AND MEDICINE, ALBERT EINSTEIN COLLEGE OF MEDICINE, AND IRVINGTON HOUSE INSTITUTE, AND DEPARTMENT OF MEDICINE, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

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Polyribosomes have been shown to be the active unit of protein synthesis in gamma globulin-producing cells from both rabbit lymph nodes[1] and mouse plasma cell tumors.[2] 7*S* gamma globulin consists of two heavy (H) and two light (L) chains linked by disulfide bonds. The availability of techniques for separating and identifying the two chains affords a unique opportunity to study the synthesis and assembly of the polypeptide chains of a disulfide-linked protein.

Mouse plasma cell tumors provide a cell population in which 20–30 per cent of the newly made protein is homogeneous gamma globulin. With these tumor cells it has been possible to determine (1) whether the two chains are made on polyribosomes of different sizes, (2) whether the two chains are assembled on polyribosomes, (3) the relative rates of synthesis of the two chains, and (4) the approximate size of the messenger RNA's.

Materials and Methods.—Two transplantable mouse plasma cell tumors were used. Cells from the MPC-11 tumor (provided by Dr. John Fahey) produce approximately four times as many L as H chains. The B-J tumor (provided by Dr. Elliot Osserman) synthesizes L chains, but few if any H chains. Immunologic and electrophoretic analysis indicated that these two polypeptides represented 20–30% of the total protein synthesized by both tumors.

Cells were teased from the tumors, filtered through a stainless steel screen, washed four times, and resuspended in Eagle's medium[4] containing $^1/_{100}$ the normal amounts of amino acids.[3] After adjusting the cell concentration to $1–2 \times 10^7$ cells per ml and preincubating at 37° for 15 min, the cells were exposed to various mixtures of C^{14}-amino acids at 10–20 μc/ml. Incorporation of the radioactive precursors was stopped by adding 10 vol of chilled Earle's saline, after which 10^7 cells were washed three times in cold Earle's saline and resuspended in 1 ml of a cytoplasmic extract freshly prepared from 10^8 HeLa cells.[5] The tumor cells were then disrupted by adding desoxycholate to a final concentration of 0.5%, the cell lysates were immediately layered on 15–30% linear sucrose gradients,[6] and centrifuged for 130–150 min at 24,000 rpm in the SW25.1 swinging-bucket rotor (Spinco). Gradients were analyzed for UV absorbancy at 260 mμ and for acid-precipitable radioactivity as previously described.[3]

"Pulse-chase" experiments were performed by incubating cells for $1^1/_2$ min with a mixture of C^{14}-labeled arginine, lysine, threonine, valine, and leucine (New England Nuclear Corp., 150–300 mc/mM) at a final concentration of 10–20 μc/ml and "chasing" with a 200-fold excess of unlabeled amino acids. Samples taken at the time of addition of the chase, and at 15-sec intervals thereafter, were analyzed for polyribosome-associated acid-precipitable radioactivity. The addition of the unlabeled amino acids immediately stopped the incorporation of radioactive label into acid-precipitable material.

The identification and quantification of H and L chains (*a*) in whole-cell lysates, (*b*) as newly completed and released polypeptide chains from the tops of sucrose gradients, and (*c*) as nascent polypeptides on polyribosomes, was carried out by a modification of polyacrylamide gel electrophoresis.[7] Samples were prepared for electrophoresis by treatment with 10% acetic acid, 1% sodium dodecyl sulfate (SDS), 0.5 *M* urea, and 1% 2-mercaptoethanol (ME). Under these conditions, aggregated or insoluble proteins are solubilized so that they are neither lost nor trapped at the origin of the gel.[7, 8] The sample was then dialyzed at room temperature for 16 hr against 0.1% SDS, 0.5 *M* urea, 0.1% ME, and 0.01 *M* phosphate buffer at pH 7.1. Five per cent polyacrylamide gels were prepared in the same buffer but with a phosphate concentration of 0.1 *M*,

and electrophoresis was carried out at $3^1/_2$ v per cm for $2^1/_2$ hr. Gels were prepared for counting by automated extrusion through a small orifice and distribution onto planchettes.[7] Figure 1 shows the electropherogram of a whole-cell lysate of the MPC-11 tumor after 17 min incubation with C^{14}-amino acids. H and L chains are easily distinguishable above the background of cell proteins. Since the reduction of purified 7*S* gamma globulin from this tumor yields twice as many counts in H as L chains, it can be calculated by integration of the areas under the peaks, above background, that 3.6 L chains are produced for each H chain.

In order to determine the size of the messenger RNA molecules associated with different size polyribosomes, cells were exposed to 350 μc of tritiated uridine (14.3 c/mM, Nuclear-Chicago Corp.) for 10–15 min to label only the polyribosome-associated "messenger" RNA and none of the 28*S* and 16*S* ribosomal RNA.[9] Polyribosomes from the 190*S* and 270*S* areas of the polyribosome gradient were concentrated by centrifugation, extracted with 1% SDS, and then analyzed on sucrose gradients containing 0.5% SDS, 0.1 *M* sodium chloride, and 5×10^{-3} tris buffer pH 7.1.[10] Gradients were centrifuged at 25°C in the SW25.3 swinging-bucket rotor (Spinco) for 16 hr at 24,000 rpm, and samples were processed for acid-precipitable radioactivity and optical density as previously described.[10] Sedimentation values were converted to molecular weights using the formula of Hall and Doty for single-stranded RNA,[11] and the size of the corresponding polypeptide chain was calculated, based on the triplet code.

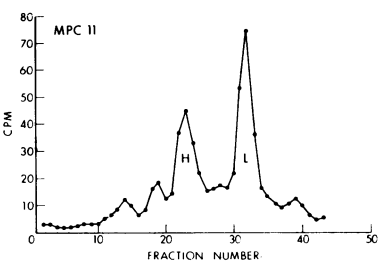

FIG. 1.—Polyacrylamide gel electropherogram of a whole-cell lysate of MPC-11 tumor after 17 min exposure to mixed C^{14}-amino acids (NEC-445). The location of H and L chains was determined by stained gels of purified reduced gamma globulin. The anode is on the right in this and subsequent electropherograms.

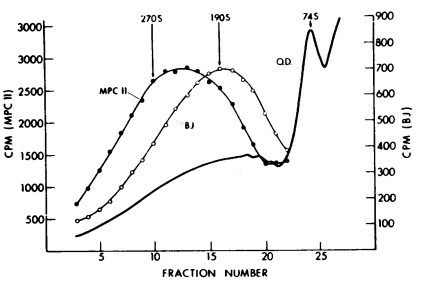

FIG. 2.—Polyribosome-associated amino acid incorporation by the MPC-11 and B-J tumors. Cells were incubated with mixed C^{14}-amino acids (NEC-445), and sucrose gradients centrifuged at 24,000 rpm for 130 min (SW25). The optical density curve (OD) is that of the carrier HeLa cell cytoplasm.

Results.—Polyribosome profiles of different tumors: The pattern of polyribosomal amino acid incorporation by tumor cells which make both H and L chains (MPC-11) differed from that in tumor cells which make only L chains (B-J) (Fig. 2). In the MPC-11 tumor, relatively more activity was associated with larger polyribosomes (>210*S*), whereas in the B-J tumor amino acid incorporation was more sharply confined to the smaller polyribosomes (170–210*S*) with a peak at 190*S*. The 250–290*S* fraction represented the greatest difference between the tumors. This observation suggests that the messengers coding for the two chains may differ in size.

Characterization of nascent polypeptide chains associated with the 190S and 270S polyribosomes: Preliminary attempts at the electrophoretic analysis of the polypeptide chains associated with polyribosomes from the 190*S* and 270*S* fractions of the MPC-11 tumor were unsuccessful because of the broad smear produced by large numbers of incomplete chains. Ribosome-associated radioactivity could, however, be progressively restricted to the completed or nearly completed nascent

polypeptide chains by analyzing polyribosome fractions from sucrose gradients of cells which had been labeled for 90 sec, and then chased for the amount of time necessary almost to complete the nascent polypeptide chain. This procedure reduced but did not eliminate the background caused by incomplete chains. Figure 3 shows electropherograms of the polypeptides associated with 190*S* and 270*S* polyribosomes after 15- and 30-sec chases. No H chains were found associated with the 190*S* polyribosomes; a distinct L chain peak was present 15 sec after the beginning of the chase and had almost completely disappeared after 30 sec. H chains were found associated with the 270*S* polyribosomes for up to 60 sec, but were undetectable thereafter (not shown in Fig. 3). These findings are consistent with the synthesis of H chains on larger polyribosomes and that of L chains on smaller polyribosomes.

The 270*S* polyribosomes also contained L chains. In contrast to the rapid synthesis and release of L chains from the 190*S* polyribosomes, the 270*S*-associated L chains persisted for as long as 90 sec after the chase, and in some experiments even longer. By this time, labeled H chains were no longer detectable, and labeled L chains had long since been chased off the 190*S* polyribosomes. This observation suggests that completed and labeled L chains in the intracellular pool continue to attach to nascent unlabeled H chains on the 270*S* polyribosomes.

Some of the polyribosome-associated polypeptide chains migrate slightly more slowly than the released and completed chains. Similar findings have been reported for polyribosome-associated poliovirus[12] and hemoglobin[13] polypeptides, as well as for hemoglobin synthesized in the cell-free system,[14] and are as yet unexplained.

Kinetics of release of polyribosome-associated radioactivity: To obtain further information about the synthesis and assembly of H and L chains, the rates of synthesis of polypeptides on different size polyribosomes was correlated with the rates of appearance of released, completed H and L chains. Cells from the MPC-11 tumor were pulsed with C^{14}-amino acids for $1^1/_2$ min and then chased with a large excess of unlabeled amino acids. As shown in Figure 4, nascent proteins were chased more rapidly from the smaller polyribosomes than from the larger ones. Most of the chaseable radioactivity was gone from the 190*S* polyribosomes by 30–45 sec, and from the 270*S* polyribosomes only after 60–75 sec, in agreement with the times required for the synthesis of L and H chains, respectively, as determined by direct examination (Fig. 3).

The ratio of radioactivity in completed and released L and H chains (Fig. 5) decreased rapidly during the first 60 sec of the chase from an initial value of 4:1 –1.5:1 (a molar ratio of 3:1), and subsequently rose to 1.8:1 between 90 and 120 sec. The initial ratio of 4:1 reflects the release into the intracellular pool of more fully labeled L than H chains during the $1^1/_2$-min pulse. The rapid fall in that ratio during the first 60 sec of the cold amino acid chase reflects the following release of relatively more of the slowly completed labeled H chains. The increased ratio of L to H chain radioactivity between 90 and 120 sec, although slight, was a reproducible finding, and is best explained by the continued release from the 270*S* polyribosome of a few residual labeled L chains associated with unlabeled H chains. The final ratio of 1.8:1 is the steady-state ratio of L to H chain counts observed in cells exposed to continuous labeling for longer periods of time (see Fig. 1).

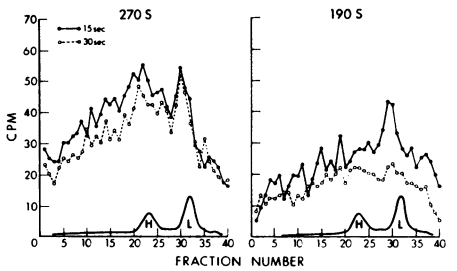

FIG. 3.—Electropherograms of nascent polypeptides associated with 270*S* and 190*S* polyribosomes of the MPC-11 tumor. Cells were labeled with C^{14}-amino acids and chased as described in the text. Polyribosomes of selected sizes were concentrated from pooled fractions of three gradients by centrifugation. The nascent polypeptides were released by 100 γ/ml of ribonuclease at 37° for 30 min, treated with SDS, urea, and ME, and analyzed electrophoretically. Marker gels prepared as in Fig. 1 were run simultaneously (———). Radioactivity of nascent polypeptides after 15-sec (●——●) and 30-sec (○- - -○) chases are shown.

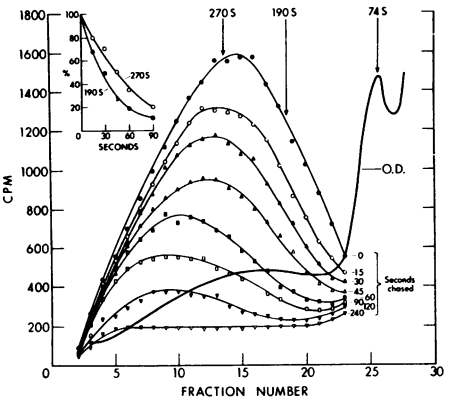

FIG. 4.—Pulse-chase analysis of MPC-11 polyribosomes. Cells were pulse-labeled for $1\frac{1}{2}$ min with five C^{14}-labeled amino acids and then chased by a 200-fold excess of unlabeled amino acids. Equal aliquots were taken at intervals from 15 to 240 sec after chasing, and analyzed for polyribosome-associated incorporation into nascent polypeptides. The insert depicts the relative rates of disappearance of radioactivity from the 190*S* and 270*S* polyribosomes after subtraction of nonchaseable counts. The finding of nonchaseable radioactivity has been noted in other systems.[19, 22]

Messenger RNA: Preparation of intact polyribosomes and the identification of the polyribosome class responsible for the synthesis of H and L chains made it possible to determine the approximate sedimentation values of the associated "messenger RNA's." The rapidly labeled nonribosomal RNA associated with 190*S* polyribosomes was larger than 9*S*, with a peak between 9 and 11*S*. That isolated from 270*S* polyribosomes, although very heterogeneous in size, was all larger than 10*S* and peaked at 14–16*S*.

Based on the formula of Hall and Doty,[11] most of the "messenger" RNA in 190*S* polyribosomes would code for a polypeptide chain of about 24,000, and the 14–16*S* RNA from 270*S* polyribosomes would code for a polypeptide of about 50,000, consistent with the reported sizes of the L and H polypeptides.[15] These calculations, however, are based on assumptions such as partial specific volume of RNA which have not been tested in our system.

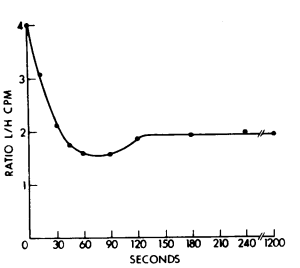

FIG. 5.—Ratio of radioactivity in completed L and H chains after chasing. The relative radioactivity in completed and released chains was determined electrophoretically on the material from the tops of each gradient shown in Fig. 4.

Discussion.—Several independent lines of evidence indicate that L and H chains are synthesized independently on two classes of polyribosomes, the L chains on polyribosomes of approximately 190*S* in 30–45 sec, and H chains on larger polyribosomes in 60–75 sec: (*i*) the difference in polyribosome profile of a tumor making

both L and H chains and one making L chains alone; (*ii*) the direct demonstration of nascent L and H chains on polyribosomes of different size; (*iii*) the correlation between the rate of completion of the two polypeptide chains with the rate of synthesis of nascent peptides on polyribosomes of different sizes. The findings of Voss and Bauer[16] of different ratios of specifically precipitable L to H chains on different size polyribosomes are consistent with the above interpretation. The demonstration of separate polyribosomal sites for the synthesis of L and H chains, implying discrete messenger RNA's rather than a single polycistronic messenger, is consistent with the nonlinkage of genetic markers for H and L chains.[17]

The L chains are synthesized in considerable excess. After their release from polyribosomes, the L chains then apparently attach to H chains while the latter are still associated with large polyribosomes. The fact that, even after short labeling times, free single H chains have not been detected in the cytoplasm of the MPC-11 tumor provides further evidence for assembly on polyribosomes. It is unlikely that the latter observation reflects the relative insolubility of the H chains, since the whole cell was treated with SDS, urea, and mercaptoethanol under conditions where no material sedimented or was trapped at the top of the gel. The rise in ratio of released labeled L to H chains occurring between 90 and 120 sec after chase (Fig. 5) is also consistent with the continuing release of labeled L chains with newly synthesized and unlabeled H chains. Finally, and most important, labeled L chains were demonstrable on the 270*S* polyribosomes even after all the nascent L and H chains had been chased off the 190*S* and 270*S* polyribosomes, respectively. The synthesis of excess L chains probably increases the efficiency of this assembly process. The finding of excess L chain synthesis and no free H chains in hyperimmune rabbit lymph node cells[18] suggests that a similar process of assembly occurs in antibody-synthesizing cells. We emphasize that this mechanism of assembly may not be obligatory, and that the attachment of L chains to nascent H chains may not be necessary for release of H chains.

Assembly on polyribosomes has been suggested for β-galactosidase[19, 23] and for hemoglobin.[13] Colombo and Baglioni[13] have suggested that β chains of hemoglobin combine with ribosome-associated and *completed* α chains before the latter can be released. This conclusion is based on their findings that there are more completed α than β chains on reticulocyte polyribosomes, and that after a short pulse, the specific activity of the β chains in newly made soluble hemoglobin is greater than that of α chains. Because the polyribosomes responsible for H and L chain synthesis can be separated by size (in contrast to hemoglobin), it has been possible to show that L chains are released and assembled onto nascent and perhaps incomplete H chains. These observations suggest an alternative hypothesis for the assembly of hemoglobin polypeptides, namely, completed and released α chains attach to nascent and incomplete β chains. This explanation is consistent with the data of Colombo and Baglioni[13] and the finding that excess released α chains are present in β-thalassemia.[20] Since excess released β chains have been reported in α-thalassemia,[21] assembly may not be necessary for the release of either chain.

Summary.—Gamma globulin synthesis in mouse plasma cell tumors occurs on polyribosomes. Light polypeptide chains are made in 30 sec on polyribosomes of approximately 190*S*, whereas heavy chains are made in 60 sec on larger polyribosomes of approximately 270*S*. Assembly appears to occur by attachment of the

free, released light chains onto the nascent heavy chains. "Messenger" RNA's of sizes compatible with the known molecular weights of light and heavy chains have been demonstrated in the corresponding polyribosomes.

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