Proc. Nat. Acad. Sci. USA
Vol. 68, No. 10, pp. 2514–2518, October 1971

Beta Thalassemia and Translation of Globin Messenger RNA

(reticulocytes/polyribosomes/alpha chains/beta chains)

DAVID G. NATHAN*†, HARVEY F. LODISH*‡, YUET W. KAN†, AND DAVID HOUSMAN*

* Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139;
† Division of Hematology of the Department of Medicine, Children's Hospital Medical Center, and the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

Communicated by Alexander Rich, August 6, 1971

ABSTRACT To define the quality and relative quantity of beta and alpha messenger RNA in human nonthalassemic and thalassemic reticulocytes, intact cells were incubated with [^{35}S]methionine. The relative amounts of beta- and alpha-nascent chains on polysomes of different sizes were measured by tryptic digestion of pooled polysomes and by determination of the specific activities of beta and alpha peptides that contain methionine. Beta-chain synthesis predominated on heavy polysomes in nonthalassemic, as well as in thalassemic cells. Since beta chains in thalassemia are made on normal-size polyribosomes, we conclude that the defect in thalassemia does not involve reduction in the rate of initiation of translation due to the production of an abnormal beta message. Such would lead to beta-chain synthesis on very small polysomes. We therefore suggest that the decreased production of beta-globin chains results from a decreased amount of functional beta-globin messenger RNA.

In beta thalassemia the rate of production of beta-globin chains is depressed. Alpha chains accumulate and form inclusions that damage the cell membrane and cause the ineffective erythropoiesis that dominates the clinical manifestations of the disease (1).

The cause of the depression of beta-chain synthesis in beta thalassemia is unknown and, in fact, may vary in the different beta-thalassemia syndromes. In general, however, two types of pathophysiologic mechanisms have been proposed (2, 3). *A*. Decreased beta-messenger RNA production due to diminished content or transcription of beta-globin DNA. *B*. Production of abnormal beta-messenger RNA, with one or more codon substitutions, which could cause a delay in initiation, elongation, or termination of the nascent beta-globin chain. The purpose of the investigations reported here was to examine these two possible mechanisms in an effort to define the pathophysiology of beta thalassemia more securely.

Since direct techniques were not available for the analysis of the structure of beta- and alpha-globin messages, a functional method was adopted for examination of the quality of the beta message in beta thalassemia. The technique was based on the studies of Hunt and Hunter (4), who observed that in rabbit reticulocytes, nascent beta-globin chains (and, therefore, beta-globin messenger RNA) are associated with larger clusters of ribosomes than are alpha-globin nascent chains. In this paper, we demonstrate the same findings in human reticulocytes.

The number of ribosomes on a message (polysome size) is directly proportional to the rate of initiation of chain synthesis and inversely proportional to the rate of chain elongation. The translation rate and termination of beta-globin chains have been found to be normal in beta-thalassemic reticulocytes (5), and the defect lies in the mRNA–ribosome complex (6). Hence, diminution of the polysome size associated with beta-globin message in thalassemic reticulocytes would indicate a reduction in the rate of initiation of the thalassemic beta-globin message. Presumably, such a reduction in initiation rate would be due to an alteration in the sequence of the message in close proximity to the initiating codon. In contrast, a predominance of nascent beta-globin chains on heavy polysomes indicates normal initiation and, presumably, normal structure of the beta message. The latter alternative appears to be the case in homozygous beta thalassemia.

MATERIALS AND METHODS

Measurement of the polysome sizes associated with the alpha- and beta-chain message in human reticulocytes demanded certain modifications of the methods used for similar analyses in rabbit reticulocytes (4). These modifications were required because human reticulocytes have fewer and, presumably, less active polysomes, and some lysates may contain sufficient nuclease activity to alter the polysome profile during sample preparation. In addition, Hunt and Hunter's technique for the detection of nascent alpha and beta chains from rabbit is not applicable in human reticulocytes because the amino-acid compositions of the two human chains are not sufficiently dissimilar. For this reason, [^{35}S]methionine of very high specific activity was used to label the nascent chains. Trypsin digestion of the nascent chains then permitted separation of the methionine-containing peptides, of which there are only one in the human beta chain, two in the alpha chain, and two in the gamma chain.

The overall procedure applied to human reticulocytes is as follows: reticulocyte-rich blood from two patients with different types of hemolytic anemia and from one patient who had been splenectomized for homozygous beta thalassemia [patient S.D. (7)] were washed three times at 4°C with isotonic Krebs–Ringer phosphate buffer (pH 7.4). The cells were resuspended in the buffer at an approximate hemotocrit of 50%. Glucose, at a final concentration of 10 mM, and a solution of essential amino acids without methionine (8) were added. The cells were incubated for 5 min at 37°C to achieve temperature equilibrium. [^{35}S]methionine (about 5000 Ci/mmol) was added at a concentration of 30–50 μCi/ml. The incubation then proceeded for 30 min. Samples of 5 μl each were removed at regular intervals,

‡ To whom to send reprint requests.

and the protein radioactivity was determined to establish the linear rate of hemoglobin synthesis. At 15 min, most of the sample was removed and immediately chilled. Cycloheximide was added, at a final concentration of 50 μg/ml, to block further ribosome movement, and the cells were washed three times in ice-cold isotonic sodium chloride, to which cycloheximide (50 μg/ml) had been added.

The cells were then divided into two aliquots. A 0.5-ml sample of a rabbit-reticulocyte lysate (9) was added to one of the aliquots. To each aliquot was then added 3 volumes of iced distilled water containing 1 mM magnesium acetate, 0.1 mM EDTA, and 50 μg/ml of cycloheximide. After gentle agitation to accomplish hypotonic cell lysis, the membranes were removed by two 10-min centrifugations at 12,000 × *g* in an angle-head rotor. 6 ml of each membrane-free lysate was layered atop a 30-ml, 20–50% linear sucrose gradient (9). Another 0.5-ml sample of the same rabbit-reticulocyte lysate was added to 5.5 ml of the gradient buffer and layered atop a third sucrose gradient tube. The tubes were then centrifuged at 26,500 rpm in a Beckman L2-65B ultracentrifuge in an SW 27 rotor for 6.5 hr at 4°C. After centrifugation, each gradient tube was pumped from the bottom to the top at a rate of 3 ml/min through a 5-mm quartz flow cell, in a Gilford recording spectrophotometer, at a wave length of 260 nm. 1-ml fractions were collected, and the radioactivities of an aliquot were measured after treatment with 0.1 M NaOH and precipitation with 5% trichloracetic acid in the presence of carrier bovine serum albumin. The radioactivities associated with polysomes of particular sizes were determined by inspection of the radioactive and absorbance profiles of polysomes. Appropriate tubes were combined into pools for further analyses of specific alpha- and beta-chain radioactivity on polysomes of various sizes.

5 mg of bovine-serum albumin and a variable amount of [^{3}H]methionine-labeled human globin were added to each polysome pool. The addition of tritium was arbitrarily determined to provide a ^{3}H/^{35}S ratio of about 5:1. After a 10-min incubation at 37°C, in the presence of 0.1 M NaOH, the protein was precipitated with a final concentration of 5% trichloracetic acid and washed twice with the Cl_3CCOOH. The protein was finally dissolved in distilled water and lyophilized. After two subsequent lyophilizations from water, the protein was incubated in 2 ml of 1% NH_4HCO_3 at 37°C with 0.1 mg of trypsin, which had been treated with the chymotrypsin-inhibitor tosylamido-phenylethyl-chloromethylketone (Worthington Biochemicals) for 4 hr, and again for 8 hr. The hydrolysate was twice lyophilized, and 0.1 ml of pyridine acetate buffer (pH 6.5) was added. The peptides were subjected to electrophoresis at pH 6.5 on Whatman 3-MM paper at 40 V/cm for about 90 min. The radioactive areas were identified by radioautography with Kodak x-ray film. 1-cm strips of the appropriate areas of the paper were cut out and incubated in 1 ml of 0.1 N KOH in scintillation vials for 45 min at 60°C. 10 ml of Aquasol (New England Nuclear Corp.) was added and the ^{3}H and ^{35}S radioactivities were determined after appropriate corrections. The amount of ^{35}S in nascent or complete alpha and beta chains in the samples was determined from the average ^{35}S/^{3}H ratio, calculated from the 3–4 highest count rates, from the alpha T5 and beta T4 peptides (Figs. 1 and 3). The beta/alpha-synthetic ratio for each pool was then established from: beta ^{35}S/^{3}H:alpha ^{35}S/^{3}H.

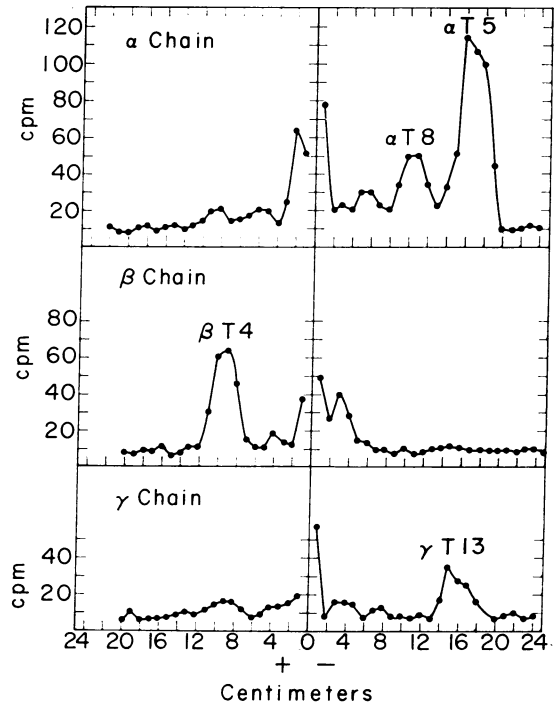

FIG. 1. Electrophoresis of trypsin digests of human alpha-, beta-, and gamma-globin chains labeled with [^{35}S]methionine. The globin chains were isolated by urea–carboxymethyl cellulose chromatography (11) from the blood of the patient with homozygous beta thalassemia after incubation with [^{35}S]methionine. The location of the peptides within the chains is indicated above each radioactive peak by the appropriate notation.

RESULTS

Adequate separation of the tryptic peptides labeled with [^{35}S]methionine from human alpha, beta, and gamma chains was essential for subsequent estimation of the particular nascent globin chains that are associated with human polysomes. Fig. 1 demonstrates that the separation procedure was satisfactory. Reticulocytes of the patient with beta thalassemia were incubated with [^{35}S]methionine, and the alpha-, beta-, and gamma-globin chains were subsequently separated by urea–carboxymethylcellulose chromatography (11). These chains were treated with trypsin and subjected to paper electrophoresis as described above. The two methionine-containing tryptic peptides of the alpha chain (alpha T8 and alpha T5) were well separated from the single methionine-containing peptide of the beta chain (beta T4). Only one of the methionine-containing peptides of the gamma chain (gamma T13) was detected, and it migrated between alpha T8 and alpha T5. Gamma T13 contamination of alpha T5 radioactivity did not present a problem in the thalassemic patient chosen for this study, since gamma-chain synthesis was shown by chromatography to equal only 10–15% of the alpha-chain synthesis in peripheral blood (11). For comparison of beta-chain and alpha-chain synthesis, only the radioactivities of alpha T5 and beta T4 were analyzed.

Fig. 2 shows the distribution of polysomes and nascent polypeptide chains in nonthalassemic human reticulocytes after incubation with [^{35}S]methionine. The second sample of nonthalassemic human reticulocytes provided similar results. The absorbance due to polysomes (fractions 4–16)

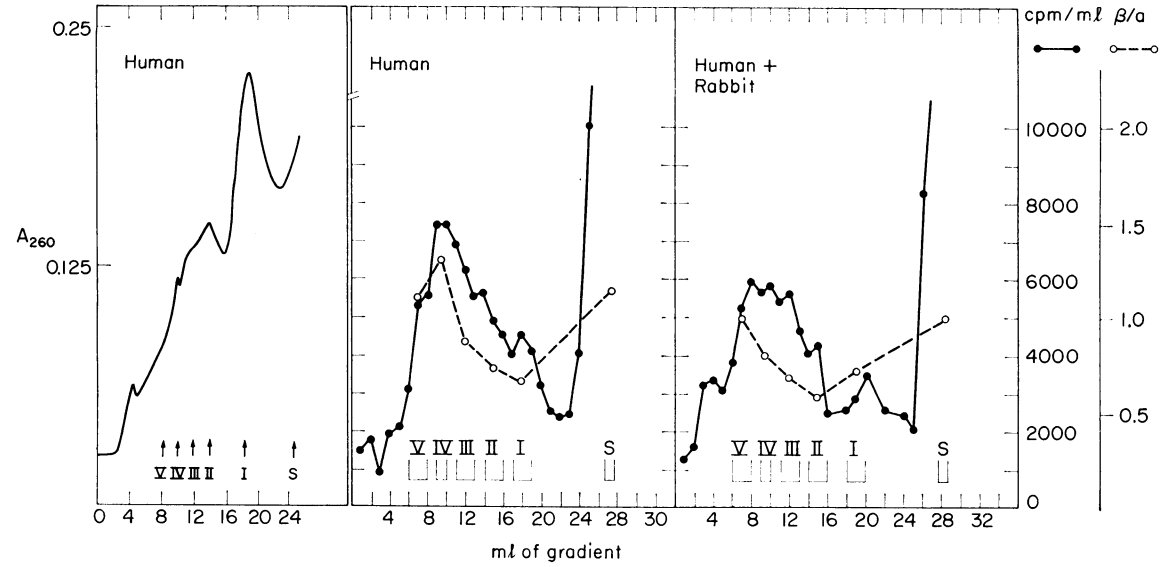

FIG. 2. Absorbance, radioactivity, and nascent-chain radioactivity profiles of polysomes from the reticulocytes of a patient with nonthalassemic hemolytic anemia. Centrifugation is from *right* to *left*. *Left panel*, absorbance at 260 nm of the polysomes after centrifugation of the lysate, with no additive, through a sucrose gradient. *Middle panel*, radioactivity associated with these polysomes and the beta/alpha nascent-chain ratio within the pools of polysomes shown by the brackets below each Roman numeral (I = monosomes; II = disomes; etc.). *Right panel*, same as *middle panel*, except that rabbit lysate was added to the human lysate before centrifugation. Cycloheximide and the low temperature prevented any synthesis by the carrier rabbit polysomes. The difference in the ratio alpha/beta in the two supernatants (1.12, *middle*; 1.00, *right*) is a measure of the error in this type of analysis.

was clearly distinguishable from that due to monosomes (fraction 18) and hemoglobin (top of gradient), but the absorbances of the various size classes of polysomes were only slightly resolved (left panel). The positions of the various polysome classes in the gradient were determined from the tube in which rabbit polysomes were added to the human lysate before ultracentrifugation. These are indicated by vertical arrows. In both gradients, the radioactive polysome profiles (middle and right panels) were quite similar to the profiles in rabbit reticulocytes (10). It is of interest that the polysomes, as measured by ultraviolet absorbance, appeared much more degraded than the active polysomes, measured by incorporation of ^{35}S radioactivity; it appears that these cells contain some inactive clusters of ribosomes. The fractions from which polysome pools of various sizes were prepared are shown by the brackets surrounding the Roman numerals in each panel.

To determine the nascent beta- and alpha-chain radioactivity within the designated polysome classes, [^{3}H]methionine-labeled human globin was added to each pool before tryptic digestion and electrophoresis. Fig. 3 provides an example of such a study of the polysome pools shown in the middle panel of Fig. 2. The $^{35}S/^{3}H$ ratios in alpha-T5 and beta-T4 peptides were nearly equal in the supernatant hemoglobin. Nascent alpha chains exceeded beta in the smaller polysome classes, but beta clearly exceeded alpha chains in the tetra- and pentasomes. The change in the beta/alpha-synthetic ratio across the polysome profile is shown by the interrupted lines and open circles of Fig. 2. In both samples of the nonthalassemic human reticulocytes, beta-chain synthesis predominated in the larger polysome classes.

After the establishment of the distribution of nascent beta- and alpha-chain synthesis in nonthalassemic human reticulocytes, the cells of the patient with homozygous beta thalassemia were similarly analyzed (Fig. 4). A small, but detectable, population of polysomes of various sizes was evident. The addition of nonradioactive rabbit polysomes to the human lysate (right panel) seemed to enhance the resolution of radioactivity in the classes of human thalassemic polysomes. Slight nuclease activity in the thalassemic lysate was suggested by the relatively high radioactivity in the monosomes and disomes, as compared to that observed in tetra- and pentasomes. However, distinct radioactive peaks indicative of the various size classes of polysomes were identified. These were pooled, and the methionine-containing peptides of nascent globin chains were separated. As expected for beta thalassemia, the beta/alpha-synthetic ratio was abnormally low in all of the polysome pools and in the supernatant hemoglobin. However, the distribution of nascent-chain radioactivity was similar to that observed in the nonthalassemic cells; there was a definite rise in the beta/alpha synthetic ratio detected as we proceeded from the lighter to heavier polysomes.

DISCUSSION

This study was designed to determine whether the reduced synthesis of beta chains in beta thalassemia is due (*a*) to a reduction in the amount of beta-globin mRNA that functions normally in beta-chain synthesis or (*b*) to a normal amount of beta mRNA that, however, is deficient in some stage of initiation of beta-globin synthesis.

The findings of Hunt and Hunter (4) in rabbit reticulocytes formed the basis of our inquiry. Although Kazazian and Freedman (12) observed only small differences in the polysome distribution of rabbit nascent beta- and alpha-chains, Hunt and Hunter clearly determined that beta-chain synthesis predominates on polysomes that are heavier

than those synthesizing alpha chains. In subsequent articles, Hunt, Hunter, and Munro (13, 14) ascribed this finding to a faster elongation rate of alpha chains, rather than to a faster rate of initiation of beta chains, but the difficulties inherent in the measurement of elongation rate did not permit this conclusion to be drawn unambiguously. Furthermore, studies in our laboratory performed on rabbit, and most recently on human, reticulocytes have provided strong evidence that the rate of initiation of translation of beta-chain messenger exceeds that of alpha-chain messenger. Equal rates of alpha- and beta-chain production are brought about by an excess of alpha messenger relative to beta in the cell (15).

Application of these findings to a functional assay of nascent beta-chain initiation in thalassemia depended upon successful modification of the techniques developed for rabbit polysome isolation to studies of human cells. First, it was necessary to recover reasonable amounts of polysomes from lysates of human reticulocytes and to reduce to a minimum the time of exposure of these polysomes to whatever nuclease activity might be present in lysates of human cells. Such activity may have contributed to the unusual profile of thalassemic polyribosomes that was observed by Bank and Marks (16), who pelleted ribosomes by centrifugation before the sucrose gradient analysis. The use of rabbit polysomes as "carrier" in human lysates provided a convenient marker for different sizes of polysomes in the sucrose gradient, and also offered a substrate for whatever nuclease activity may have been present in the human lysate. A minor modification of the sucrose gradient itself, to use 30 ml of 20–50% sucrose rather than 36 ml of 15–30% sucrose, permitted separation of polysomes from as much as 6 ml of lysate in one tube. These modifications of standard methods permitted adequate recovery of human polysomes. It was then possible to show that human, as well as rabbit (4, 15), nascent beta chains are associated with heavier polysomes than are alpha chains; presumably, translation of human-beta messengers is initiated at a more rapid rate than alpha, as is the case in rabbits (15).

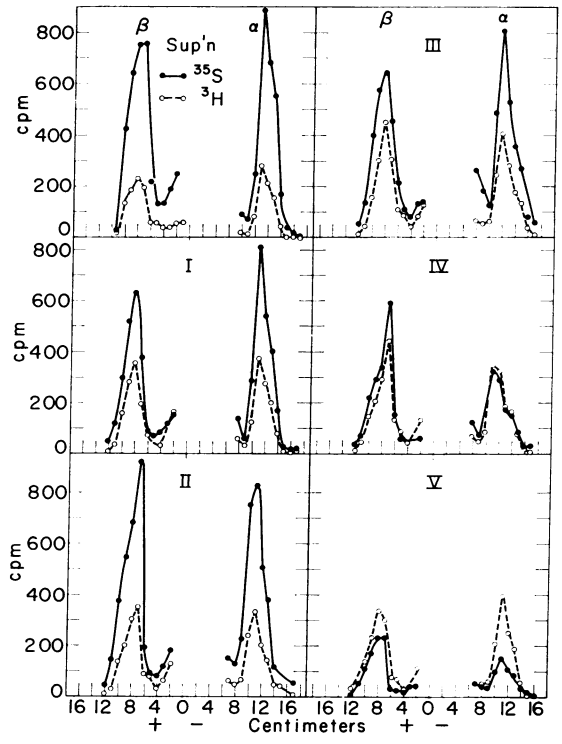

FIG. 3. Typical electrophoretic separation of [^{35}S]methionine-labeled alpha and beta-peptides derived from the pooled human polysomes shown in the middle panel of Fig. 2, together with [^{3}H]methionine peptides from carrier globin. The sizes of the polysomes are indicated by the Roman numerals in each panel. For convenience, the ^{3}H cpm are plotted as 1/10th of the actual cpm. Only the region of paper corresponding to alpha-T5 and beta-T4 peptides was counted.

The patient with homozygous beta thalassemia chosen for this study produced sufficient peripheral blood reticulocytes and hemoglobin A. Gamma-chain synthesis was only 15% of the alpha in peripheral blood cells. These necessary bio-

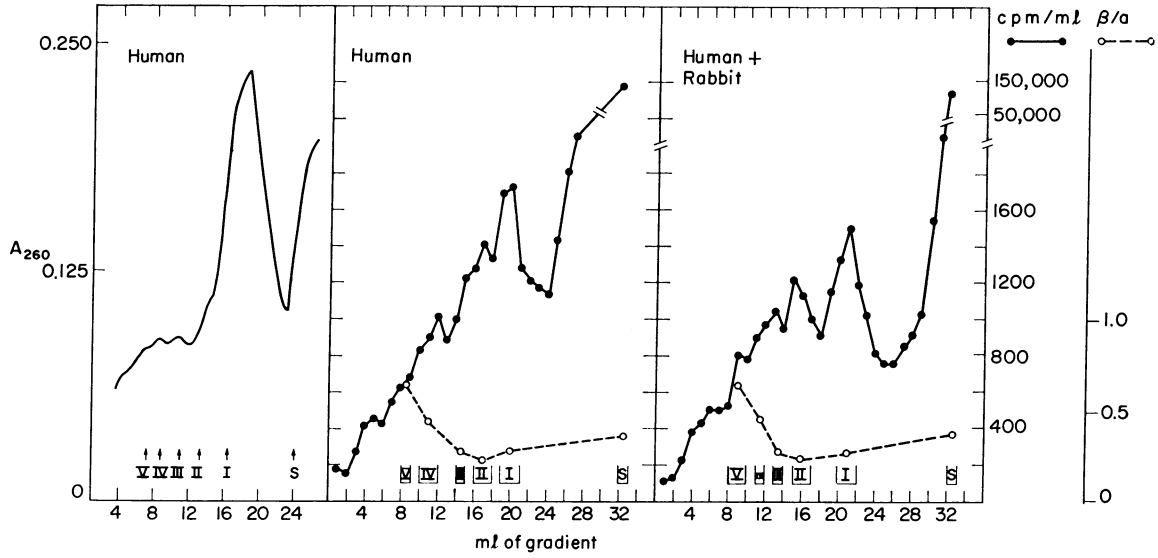

FIG. 4. Absorbance, radioactivity, and nascent-chain profiles of polysomes from the reticulocytes of a patient with homozygous beta thalassemia. See legend to Fig. 2 for details.

logical qualities, together with the availability of [^{35}S]methionine of very high specific activity, led to successful labeling of the thalassemic polysomes and the detection on them of the normal distribution of nascent beta- and alpha-chains. This study, combined with the knowledge that the elongation rate and termination of beta globin is normal in beta thalassemia (5), strongly indicate that the rate of initiation of beta messenger proceeds normally in this disease. Thus, our data do not suggest a structural abnormality of beta messenger leading to delayed initiation. Instead, we conclude that beta thalassemia is caused by a decrease in the amount of normal beta messenger RNA in erythropoietic cells. Whether this is due to decreased synthesis or decreased stability of beta mRNA is not known.

NOTE ADDED IN PROOF

Weatherall and Clegg [*Biochem. J.*, **119**, 68p (1970)] have recently demonstrated that α- and β-chains from non-thalassemic individuals are synthesized on different-sized polysomes.

This work was supported by USPHS grants no. AI-08814 and no. HD-02777 and by grants from the American Cancer Society no. ACS-E-559, The John A. Hartford Foundation, and The Charles H. Hood Dairy Foundation. Drs. Nathan and Lodish are recipients of USPHS Career Development Awards no. K3AM 35361 and no. 1KM-GM-50, 175-01, respectively. Dr. Nathan was a Visiting Scientist in the Department of Biology, Massachusetts Institute of Technology. We thank Marion Jacobsen and Andrée Dozy for their excellent technical assistance.

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