RNA Sequences Homologous to the 3′ Portion of Immunoglobulin α-Chain mRNA in Thymus-Derived Lymphocytes†

Richard I. Near* and Ursula Storb

ABSTRACT: Previous findings of immunoglobulin κ-chain RNA sequences in thymocytes (Storb, U., et al. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2467) encouraged an analogous study of immunoglobulin heavy-chain RNA. We have, therefore, examined thymocytes for the presence of immunoglobulin α-chain RNA sequences. RNA was extracted from HOPC 2020 myeloma microsomes and used to prepare α-chain messenger RNA (α-mRNA). α-mRNA was then purified to 87% purity as determined by its hybridization kinetics with α-chain complementary DNA (α-cDNA) compared with mRNA standards of known complexity. The α-mRNA was characterized by in vitro translation in a rabbit reticulocyte lysate system and found to produce two major immunoprecipitable products of 57 500 and 53 500 daltons. The α-cDNA probe which corresponded to the 3′ untranslated region and only a relatively small portion of the c region of α-mRNA revealed the presence of α-RNA sequences in thymus RNA at about 35 molecules per cell as judged by hybridization kinetics. The ratio of α to κ RNA sequences was 0.49 and 0.84 in thymus and spleen, respectively. The thermal stability of α-cDNA hybrids with thymus RNA was nearly as great as that of α-cDNA–α-mRNA hybrids with only 3% mismatching and was equal to that of κ-cDNA–thymus RNA hybrids. Cytoplasmic thymus RNA contained α-RNA sequences at approximately one-third the concentration present in thymus whole-cell RNA preparations suggesting that this RNA could function as message. Controls indicated that the α-cDNA probe did not hybridize to non-α-chain producing myeloma RNAs at long hybridization times. The results are consistent with the thymus containing α-RNA coding for an α-chain-like protein which would possibly function as a thymocyte antigen receptor.

Lymphocytes of the immune system have been classified into two divisions, thymus-derived lymphocytes (T cells or thymocytes) and bone-marrow-derived lymphocytes (B cells) (Katz & Benacerraf, 1972). Both of these cell types exhibit specificity to the antigen used to initiate various immune responses (Basten et al., 1971). The B-cell immunological specificity is mediated by immunoglobulin (Ig[1]) receptors in its surface membrane with the same specificity as its secreted Ig (Warner, 1974; Ashman, 1973; Marchalonis et al., 1972). T cells have antigen receptors which exhibit specificity in helper function (Basten et al., 1971; Lesley et al., 1971), cellular immunity (Cantor & Asofsky, 1970), and suppressor function (Gershon, 1973). However, whether the T-cell receptor contains Ig is still controversial when examined by different laboratories using the same techniques such as immunofluorescence, radioimmunoassays, cytotoxicity assays, or radiolabeling of surface proteins followed by immunoprecipitation and $NaDodSO_4$ gels (Warner, 1974; Marchalonis, 1975). Such assays may suffer from the nonspecificity of antisera used or binding of antisera to cell surfaces not via their binding sites but by such mechanisms as Fc cytophilic binding (Vitetta & Uhr, 1975). Using such assays, some investigators have found κ chain and Ig heavy chain (μ being the most common but α also being reported) on thymocytes (Hämmerling, U., et al., 1976a,b; Moroz & Lahat, 1974; Cone & Marchalonis, 1974). It has also been suggested that the heavy chain found on thymocytes may be an Ig unique to thymocytes (often referred to as IgT; Cone, 1977).

We have previously circumvented the problems associated with the above protein-biochemical techniques by working at the nucleic acid level. Using a κ-chain cDNA copy of κ-mRNA as a hybridization probe, we have demonstrated the presence of κ-RNA sequences in thymocytes (Storb et al., 1976, 1977; Storb, 1978). The κ-RNA sequences are present in the thymus cytoplasm (Storb et al., 1977) and mRNA from thymocytes translates a κ-chain product in vitro (Putnam et al., 1977). These κ-RNA sequences are present only in lymphoid cells and are not a general product made in small amounts in many cell types (Storb et al., 1977). These data suggested that the κ-RNA sequences may act as a functional message in thymocytes. There still remains the question why thymus T cells are generally not positive when tested in immunofluorescence with anti-κ antisera prepared against the c region of κ chains. It is possible that κ-RNA is less efficiently translated in T cells than in B cells or alternatively that posttranslational modifications or the cellular localization of T cell κ chains diminishes their reactivity with anti-κ antisera.

To examine thymocytes for Ig heavy chain RNA, it was necessary to purify heavy chain RNA. Using techniques similar to those used by other investigators (Marcu et al., 1978; Ono et al., 1977), we have purified Ig α-chain mRNA to 87% purity and found it to be translationally competent. An α-cDNA copy of the α message served as a hybridization probe to quantitate α-RNA sequences in thymocytes. Further, sequence homology between the thymocyte α-RNA and α-mRNA was examined. The presence of α-RNA sequences in thymocytes supports the possibility that Ig heavy chain may be used as a component of the thymocyte antigen receptor.

Experimental Procedures

Myelomas and Mice. The HOPC 2020 ($\alpha\lambda_1$), PC 3741 ($\mu\kappa$), and PC 2880 ($\gamma 2b\kappa$) myelomas were kindly supplied by Dr. M. Weigert. These tumors were serially passaged in BALB/c mice (Simonson) by subcutaneous injections of a tumor cell suspension in Dulbecco's modified Eagle's medium supplemented with 10% heat-activated horse serum. The

† From the Department of Microbiology and Immunology, University of Washington, Seattle, Washington 98195. *Received September 14, 1978; revised manuscript received December 4, 1978.* This work was supported by National Institutes of Health Research Grants AI-10685 and DE-02600. Richard I. Near was supported by a National Research Award from the National Institutes of Health (GM-07270).

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; cDNA, complementary DNA; R_0t, product of the concentration of nucleotide sequence of RNA and time of incubation (moles of nucleotides × second/liter); $R_0t/2$, R_0t value at 50% hybridization; Ig, immunoglobulin; T_m, temperature of 50% thermal denaturation; EDTA, ethylenediaminetetraacetic acid.

0006-2960/79/0418-0964$01.00/0

myelomas 66.2 and NP-2 (variants of MPC-11) were obtained from Dr. M. Scharff and passaged in mice as above. S178A myeloma (λ_1) was obtained from the Salk Institute.

Preparation of Single-Cell Suspensions from Spleen and Thymus. Thymuses for single-cell suspensions or whole-thymus tissue RNA preparations were very carefully dissected from BALB/c mice between 1 and 1.5 months of age. Great care was taken to avoid any surrounding tissue (some of which contains lymph nodes). Thymus cells were expelled by placing the thymus between the frosted ends of two glass slides, cutting the thymus into three or four slices, and gently pressing the slides together and apart while touching the frosted ends to the medium (RPMI plus 1% heat-inactivated fetal calf serum). This action draws medium up between the slides and then pushes the liquid back out. The connective tissue remains adhering to the slides while the thymocytes are released into the medium. The suspension obtained was passed through a fine nylon screen and the cells were spun down at 800*g* for 10 min. Thymocytes prepared in this manner were over 95% viable and contained no connective tissue. These thymocytes were used for whole-cell or cytoplasmic RNA preparations as described below. A small aliquot of the cell suspension was analyzed by immunofluorescence using a rabbit anti-mouse κ-chain antiserum labeled with rhodamine. The technique for cytoplasmic staining has been previously described (Clagett & Weigle, 1974). Using this technique, we detected less than 0.02% κ-chain-containing plasma cells in thymocyte suspensions.

Spleens were dissected from the same mice from which thymuses were removed. Spleens were used whole or as a single-cell suspension for RNA preparations. To prepare single-cell suspensions, spleens were put into medium (see above), an incision was made in one end of each spleen, and two bent 18-gauge needles were used to push cells from the spleenic capsule much as one would push toothpaste from its tube. Cells were passed through a nylon screen and spun down at 800*g* for 10 min. Approximately 60% of these cells were viable.

Preparation of Whole-Cell RNAs. When single-cell suspensions were used, a proteinase K procedure was used (Hilz et al., 1975). If whole organs were used, either the proteinase K or a guanidine hydrochloride technique was used (both techniques gave RNA which hybridized to α-cDNA with the same kinetics (R. Near, unpublished experiments)).

For the proteinase K technique, 15–20 thymuses or spleens or their cells were added to 40 mL containing 0.75% $NaDodSO_4$, 7.5 mM Tris, pH 7.5, 4 mM EDTA, and 50 μg/mL of proteinase K (E. M. Laboratories) in a Dounce homogenizer. The mixture was immediately homogenized with a motor-driven Teflon pestle at ambient temperature for eight to ten strokes. This was followed by four to five hand-delivered strokes of a Dounce B pestle. The homogenate, still in the homogenizer, was placed into a 40 °C water bath for 1 h during which time the mixture was given occasional strokes with a B pestle. To this mixture was added 0.25 volume of a 5× buffer (5× SD) containing 100 mM sodium acetate, pH 5.2, 1 M LiCl, and 2.5% $NaDodSO_4$. Phenol (0.5 volume equilibrated with 1× SD) was added, the mixture was vigorously shaken, and then chloroform (same volume as phenol) was added with subsequent shaking. The extraction mixture was placed into a 60 °C water bath for 3–5 min with continuous shaking. After cooling to 4 °C, the mixture was centrifuged at 2200*g* for 12 min. The aqueous phase was reextracted with phenol–chloroform at room temperature and, finally, with chloroform alone (0.5 volume). RNA was precipitated in 2.5 volumes of 95% ethanol overnight. The precipitate was dissolved in 10 mM Tris, pH 7.5, 10 mM $MgCl_2$ to about 1 mg/mL of nucleic acids and DNase (Schwarz/Mann) was added to 20 μg/mL. After 10 min at 37 °C, $NaDodSO_4$ was added to 0.5% and the mixture was phenol extracted as above (but at room temperature and with no SD buffer). RNA was precipitated by adding an equal volume of 4 M LiCl and allowed to remain at −20 °C for 1 day. RNA was spun down at 13800*g* for 12 min, redissolved in glass-distilled water, and reprecipitated by adding sodium acetate to 0.2 M and 2.5 volumes of ethanol. The precipitate was dissolved in glass-distilled water and, at this time, contained less than 3% DNA as judged by diphenylamine assays (Burton, 1956). About 180 μg of RNA is obtained per thymus. The RNA is slightly degraded (as judged by the ratio of 28 and 18S RNA peaks in sucrose gradients) from the "DNase" treatment but is adequate for hybridization purposes.

For the guanidine hydrochloride preparation, 15–20 thymuses or spleens were placed in a homogenizer with 40 mL of a solution containing 7.3 M guanidine hydrochloride (Sigma), 10% 2-mercaptoethanol, 176 mM $MgCl_2$, 1 mg/mL of heparin, 150 mM KCl, 10 mM iodoacetate, 10 mM Tris, and 1 mM spermidine. The mixture was immediately homogenized as described above. The homogenate was made 200 mM in potassium acetate and 0.5 volume of 95% ethanol (freezer-cold) was slowly added with stirring. A precipitate was allowed to form over a 3-h period at −20 °C. The precipitate was centrifuged at 4100*g* for 10 min, the supernatant discarded, and the precipitate transferred to a homogenizer with 20 mL of 1× SD buffer. The precipitate was homogenized with a motor-driven Teflon pestle until the pellet was finely suspended in solution. This solution was extracted with phenol–chloroform as described for the proteinase K procedure (except that in the first extraction 0.75 volume of phenol to 0.25 volume of chloroform was used). RNA was precipitated from the aqueous layer by adding an equal volume of 6 M sodium acetate and putting the solution at −10 °C overnight. The RNA was pelleted at 13800*g* for 15 min, redissolved in 200 mM potassium acetate, and reprecipitated overnight with 2.5 volumes of 95% ethanol. The precipitate was dissolved in a small volume of glass-distilled water to a concentration of about 10 mg/mL. RNA prepared in this manner was undegraded and contained less than 2% DNA as determined by diphenylamine. This procedure is a slightly modified version of a procedure developed by Putnam (Putnam et al., 1977; Putnam, unpublished results).

Preparation of Cytoplasmic RNA. Single-cell suspensions of thymus in 150 mM NaCl, 10 mM Tris, pH 7.5, were treated with 0.25% of both Triton X-100 and deoxycholate at 4 °C. The mixture was homogenized with three slow strokes of a Dounce B pestle and allowed to stand for 3–5 min. Nuclei were pelleted onto a 63% sucrose cushion at 1500*g* for 15 min. The supernatant was phenol–chloroform extracted as described for proteinase K. The aqueous layer was ethanol precipitated, reprecipitated in 2 M LiCl, and then reprecipitated in ethanol. The RNA was dissolved in glass-distilled water and stored at −20 °C until needed.

Purification of α-mRNA and κ-mRNA. Microsomal RNA was prepared from HOPC 2020 myeloma essentially as described (Storb, 1972; Storb & Marvin, 1976) except (1) 7 mM 2-mercaptoethanol, 20 μg/mL of polyvinylsulfuric acid, and 2 μg/mL of cycloheximide were added to the buffers (Marcu et al., 1978); (2) heparin (250 μg/mL) was added when the microsomes were pelleted onto a 63% sucrose cushion (Marcu et al., 1978); (3) microsomes were pelleted only once onto the

sucrose cushion; and (4) RNA was extracted using the same phenol–chloroform procedure as described for proteinase K. RNA was precipitated in 2.5 volumes of 95% ethanol. The RNA was dissolved in glass-distilled water, heated to 60 °C for 5 min, cooled on ice rapidly, made 10 mM Tris, pH 7.5, 500 mM KCl, 500 (or less) μg/mL of RNA, and loaded onto a 5-mL oligo(dT)-cellulose column (Collaborative Research, Inc., T3). Poly(A)-RNA (A^+RNA) was eluted using 10 mM Tris, pH 7.5 (Swan et al., 1972), and precipitated in 200 mM potassium acetate/2.5 volumes of ethanol. The A^+RNA was put through the column a second time in the presence of Me_2SO under conditions designed to dissociate aggregated RNA at a concentration of 5 μg/mL of A^+RNA (Bantle et al., 1976). The eluted A^+RNA was again precipitated as above. A^+RNA was then subjected to polyacrylamide gel electrophoresis in 98% formamide (Maniatis et al., 1975) in 0.6 × 10.0 cm tube gels. Gels were stained using ethidium bromide (1–5 μg/mL) and bands visualized under ultraviolet light. Both heavy and light chain bands were excised and homogenized in a buffer containing 0.05% $NaDodSO_4$, 1 mM EDTA, 500 mM NaCl, and 10 mM Tris, pH 7.5. The homogenate was allowed to stand at 4 °C overnight, and the acrylamide pieces were spun down at 16000*g* for 10 min. The supernatant was saved and the pellet was reextracted as above except the homogenate was only allowed to stand for 1 h. The second and first supernatants were combined and the A^+RNA was purified on a small oligo(dT)-cellulose column. The RNA was ethanol precipitated and redissolved in a small amount of glass-distilled water to a concentration of 300–500 μg/mL. These solutions were stored at −70 °C until needed.

κ-mRNA was purified from a variant of PC 3741 which produces no Ig heavy chain (R. Near, unpublished experiments). The technique of purification was as described above, except the κ-mRNA was fractionated on preparative sucrose gradients (Marcu et al., 1978) before being run on formamide gels.

κ-mRNA from MOPC-41 was purified as already described (Wilson et al., 1978).

Cell-Free Protein Synthesis. The reticulocyte lysate system described by Palmiter (1973) was used with only minor modifications. Rabbits weighing 2.0 to 2.5 kg were given five daily injections of 2.5% (w/v) phenylhydrazine at 0.275 mL per kg (Palmiter, personal communication). Rabbits were bled on the seventh day by cardiac puncture. The lysates were treated with micrococcal nuclease in order to destroy endogenous mRNA (Pelham & Jackson, 1976) and kept at −70 °C until needed. Freeze-thawing of lysates was avoided when possible. Hemin was stored as a 6.2 mM solution in 85% ethylene glycol (Adamson et al., 1969), 100 mM Tris, pH 7.5, at −70 °C and used at a final concentration of 25 μM. [^{35}S]Methionine was obtained from the Amersham Corp. at approximately 1100 Ci/mmol and used at a final concentration of 25 or 250 μCi/mL (as noted in the figure legends). Reactions were incubated at 26 °C for 100 min at volumes of 100 or 50 μL. After this period, 5-μL aliquots were removed and placed into 1 mL of glass-distilled water containing 2.5 mM cold methionine. These solutions were trichloroacetic acid precipitated as suggested (Pelham & Jackson, 1976) using a 0.33 M NaOH and 0.167 M H_2O_2 incubation followed by adding a saturating amount of Cl_3CCOOH. Before Cl_3CCOOH was added, a 10-μL aliquot was removed and spotted directly onto filters to determine total counts. Precipitate was collected by filtration onto glass fiber filter disks (Schleicher and Schuell Inc.), washed with 5% Cl_3CCOOH, and dried under an infrared lamp. The filters were counted in a toluene cocktail (Omnifluor, New England Nuclear) in a Packard Model 3320 counter. The remainder of the translation was kept at −20 °C and used for immunoprecipitation or directly placed onto $NaDodSO_4$–polyacrylamide gels.

A staphylococcal protein A–antibody system (Kessler, 1975, 1976) was used to immunoprecipitate various translation products. Anti-α-chain antiserum (from goats) was obtained from Bionetics and used at a ratio of 20 μL of antiserum to 30 μL of translation mixture. Approximately 60 000 cpm (net counts, corrected for background translation without exogenous mRNA being present) was used for each immunoprecipitation.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Cell-free translation products (about 10 000 cpm per slot), immunoprecipitates, and cold myeloma markers were run on 10% acrylamide–0.1% bis(acrylamide) discontinuous $NaDodSO_4$ slab gels (Laemmli, 1970) using a Bio-Rad Model 220 slab gel apparatus. Gels were stained with Coomassie blue, Me_2SO impregnated for fluorography (Bonner & Laskey, 1974), and exposed to preflashed Kodak X-Omat R X-ray film at −70 °C (Laskey & Mills, 1975).

cDNA Synthesis and Purification. α- and κ-cDNAs were prepared from α-mRNA and κ(3741)-mRNA templates using the reverse transcriptase RNA-dependent DNA nucleotidyltransferase of avian myeloblastosis virus system (Verma et al., 1972) modified as previously described (Storb et al., 1977), except (1) no actinomycin D was used; (2) incubation was for only 1 h; and (3) KOH treatment was at 67 °C for 12 min. This protocol does not result in complete transcripts of the mRNAs. We wished to exclude the v region components in order to eliminate problems of variability of the sequence. The α-cDNA was further purified by back-hybridization to its template α-mRNA for 0.02 R_0t unit using techniques previously described (Storb et al., 1977). The κ-cDNA was further purified by hybridization to κ-(MOPC-41)-mRNA to a R_0t of 0.02.

The size of the cDNAs was determined electrophoretically with λ phage cleaved with *Hin*II and *Hin*III as size markers (Maniatis et al., 1975). The [^{3}H]cDNA and λ pieces were run on 3.5% Tris–borate slab gels and were visualized with ethidium bromide or (for [^{3}H]cDNA) positions were determined by autoradiofluorography as described for $NaDodSO_4$ gels followed by densitometer tracing of the developed film.

[^{3}H]cDNA, RNA-Excess Hybridizations, and Thermal Denaturation of Hybrids. These methods have been reported elsewhere (Storb et al., 1976). Hybrids formed in RNA excess in 0.24 PB at 67 °C were removed at various time points and analyzed by S_1 nuclease digestion. Melts were performed by heating hybrids for 8 min at each temperature point and removing samples at 5 °C intervals.

Results

Purification and Cell-Free Translation of α-Chain mRNA. A^+RNA from HOPC 2020 microsomal RNA which had been cycled twice through oligo(dT)-cellulose displayed two major bands when electrophoresed in 98% formamide–polyacrylamide gels (Figure 1). These peaks were approximately 17–18.5 S (about 1900 nucleotides) and 13–14 S as compared with marker gels. These peaks were excised from the gels, passed through oligo(dT)-cellulose, and then translated in a mRNA-dependent reticulocyte lysate system. Figure 2 (A and B) shows the translation and immunoprecipitation of the putative α-mRNA. Note that two bands, 53 500 and 57 500 daltons, are the major products of translation and are both immunoprecipitated with a goat anti-α-chain antiserum (Figure 2B, well no. 4), while smaller products are not immunoprecipitated. A control of normal rabbit serum shows

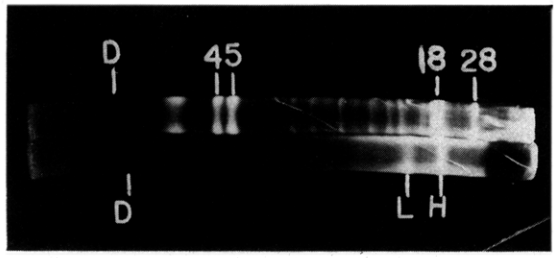

FIGURE 1: Formamide gel electrophoresis of HOPC 2020 A$^+$RNA. HOPC 2020 A$^+$RNA from two cycles of oligo(dT)-cellulose chromatography was run on 3.5% acrylamide, 98% formamide gels (30 μg of RNA per gel) and compared with a marker gel containing HOPC 2020 microsomal RNA not binding to oligo(dT)-cellulose (about 50 μg per gel). Upper gel: marker RNAs (D, dye front; 4, 5, 18, and 28 refer to *s* values of the indicated bands). Lower gel: HOPC 2020 A$^+$RNA (L, light chain (λ); H, heavy chain (α)).

that no nonspecific precipitation occurs (Figure 2B, well no. 5).

Translation gels can be misleading when only small amounts of radioactivity are loaded onto the gel. Figure 2C displays a cell-free translation at 250 μCi/mL where about three times as many cpm were loaded per gel slot as compared with the gel in Figure 2A,B. The purpose of this gel was to determine if the α-mRNA was contaminated with λ-mRNA since HOPC 2020 produces both α and λ chains. For example, we have found that γ(2b)-mRNA prepared from the myeloma PC2880 ($IgG_{2b}k$) translates into some immunoprecipitable κ-chain protein using both direct and staphylococcal precipitation techniques (R. Near & D. Putnam, unpublished results). Translation of λ-mRNA from HOPC 2020 displayed a prominent band (Figure 2C, well no. 3), which was absent from the α-mRNA translation products. This indicates no contamination with λ-mRNA. There are several bands smaller than α visible in the translation products of α-mRNA (Figure 2C, well no. 2) which are possible premature termination products and the α doublet is not resolved.

Characterization of the cDNA Probes. An α-cDNA probe was synthesized and purified by back-hybridization to its α-mRNA template (see Experimental Procedures). This α-cDNA was sized on a 3.5% acrylamide gel with λ phage *Hin*II+III cleavage markers (Maniatis et al., 1975). A densitometer tracing of the gel fluoroautoradiograph is shown in Figure 3. The gel shows a range of sizes from about 75 to 800 bases. The range contains several peaks which were also visually observed on the fluoroautoradiograph (data not shown). This cDNA is likely to consist mostly of the untranslated region but little of the constant region of the α-mRNA. Since the length of the 3′-untranslated region has not been reported for this mRNA, we cannot rigorously calculate the percent constant region. However, Honjo has recently reported the 3′-untranslated region of MOPC-31C γ_1-mRNA to be about 500 bases (Honjo & Kataoka, 1978). If this were true of α-mRNA, our α-cDNA would contain 2–3% of its radioactivity in constant region sequences (if the 3′-untranslated region was about 250 bases, the α-cDNA would have 22% of its radioactivity in constant region sequences).

Figure 4 displays the hybridization kinetics of α-cDNA hybridized to α-mRNA, λ(HOPC 2020)-mRNA, and λ-(S178A)-mRNA. Over 95% of the α-cDNA radioactivity hybridizes with the α-mRNA with monophasic kinetics over a range of less than two orders of magnitude and with a $R_0t/2$ of 2.0×10^{-3}. Comparing this $R_0t/2$ with that of a highly purified κ(PC 3741)-mRNA standard of 1.1×10^{-3} (Table I, experiment 1) yields an estimated purity of 87% (using 1200

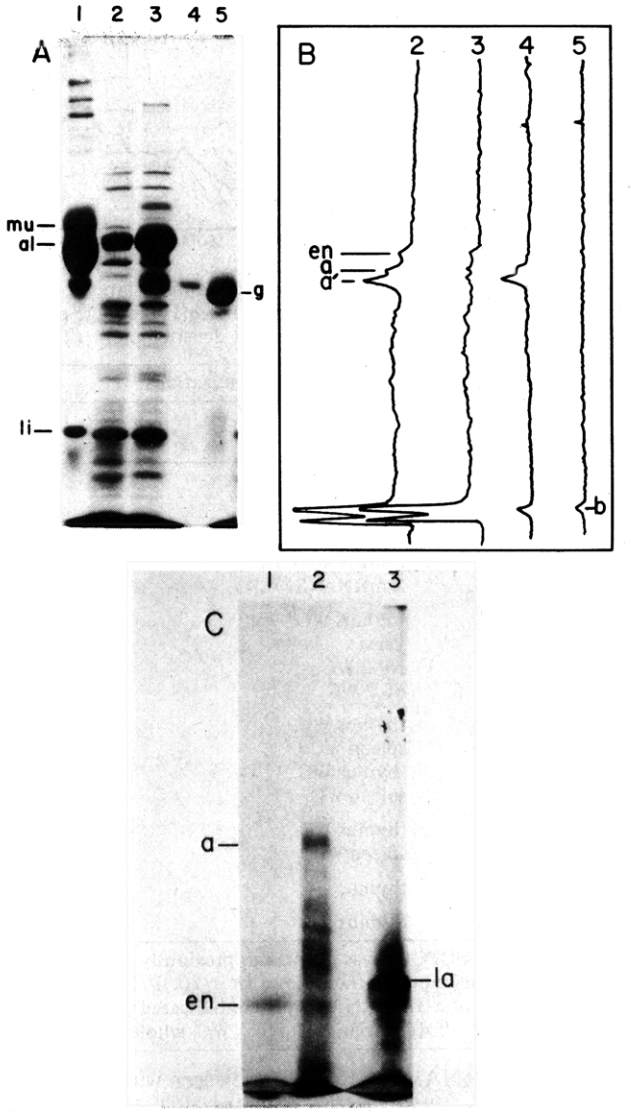

FIGURE 2: 10% NaDodSO$_4$–polyacrylamide gel electrophoresis of cell-free products and immunoprecipitable products. (A and B) These figures represent the same gel. A shows the visible stained proteins and B shows densitometer tracings of the fluoroautoradiograph of translated proteins. Molecular weights used as markers were as follows: Ig μ chain (PC 3741), 74 000 (R. Near, unpublished data); mouse serum albumin (a1), 68 000 (migrates in the same position as bovine serum albumin also at 68 000 (Weber & Osborn, 1969)); rabbit γ-globulin (g), 53 000 (Small & Lamm, 1966); κ chain (PC 3741) (li), 24 500 (R. Near, unpublished data). Slots contained the following samples: (slot no. 1) serum from PC 3741 myeloma-bearing mice; (slot no. 2) cell-free translation of α-mRNA; (slot no. 3) supernatant of the translation product after immunoprecipitation with goat anti-mouse α antiserum and *S. aureus*; (slot no. 4) immunoprecipitate with goat anti-α of the α-mRNA translation product; (slot no. 5) control precipitate using normal rabbit serum and *S. aureus*—more γ-globulins bind to *S. aureus* in A5 than A4 because nonantigen bound γ-globulins of rabbits have a greater affinity to Staph A than those of goats (R. Near, unpublished results). [^{35}S]Methionine was used at 25 μCi/mL in the translations. (A and a′ refer to the two major translation products of α-mRNA; en is an endogenous translation product; b is the bottom of the gel). (C) Fluoroautoradiograph of the cell-free translations of HOPC 2020 α- and λ-mRNAs done at 250 μCi/mL of [^{35}S]methionine. Slots contained the following samples: (slot no. 1) no exogenous mRNA was added to this translation; therefore, this represents background translation by the lysate itself; (slot no. 2) translation of α-mRNA; (slot no. 3) translation of λ-mRNA. (a = α chain; en = endogenous translation product; la = λ chain.)

nucleotides as the complexity of κ-mRNA and 1900 nucleotides for α-mRNA (see above)). The α-cDNA probe is not contaminated with λ sequences since it does not hybridize

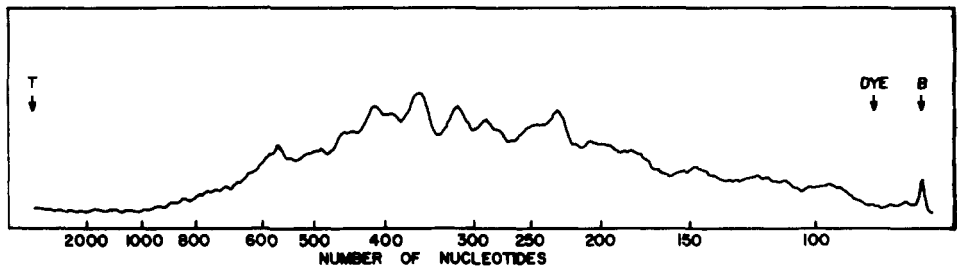

FIGURE 3: Sizing of α-cDNA in a 3.5% acrylamide slab gel. α-cDNA (about 20 000 counts per min) was loaded onto a Tris–borate 3.5% slab gel (Maniatis et al., 1975). *Hin*II and *Hin*III cleavage products of λ phage served as size markers. A fluoroautoradiograph was made of the gel and the film was scanned with a densitometer. (T, top of the gel; B, bottom, dye, bromphenol blue dye front.)

Table I: Summary of κ- and α-cDNA Hybridizations

expt no.	RNA	cDNA	$R_0t/2$	% hybridized	α/κ[b]	spleen/ thymus[c]
1	κ(MOPC-41)[a]	κ	1.1×10^{-3}	99		
	yeast	κ		0.2		
2	α-mRNA	α	2.0×10^{-3}	97		
	λ-mRNA (H2020)	α	1.6×10^{-2}	96		
	λ-mRNA (S178A)	α		12		
3	thymus WC[d]	α	330	100		
	yeast	α		7.4		
	NP-2 WC	α		17		
	66.2 WC	α		17		
4	thymus WC	α	420	~100	0.49	3.5
	spleen WC	α	120	~100	0.84	
	thymus WC	κ	130	~100		2.0
	spleen WC	κ	65	~100		
5	thymus WC	α	310	~70		1.2
	spleen WC	α	270	~70		
6	thymus cy[d]	α	900	100	0.26	
7[e]	thymus cy	κ	150	80		

[a] κ-mRNA from MOPC-41 was purified as previously described (Strob et al., 1977). [b] The ratio of α- to κ-RNA sequences in the RNA indicated was calculated using the ratio of $(R_0t/2(\kappa))/R_0t(\alpha)$ and corrected for the different complexities of α and κ by the ratio 1900/1200 bases. [c] The ratio of α- or κ-RNA sequences indicated in spleen relative to thymus was calculated by the ratio R_0t(thymus)/R_0t(spleen) for the cDNA used. [d] Abbreviations used: WC, whole cell; cy, cytoplasmic. [e] Data for experiment 7 were taken from Storb et al. (1977).

with λ(S178)-mRNA. The hybridization seen with λ(HOPC 2020)-mRNA is likely attributable to some breakdown products of α-mRNA which represent 12% contamination of the λ-mRNA band excised from formamide gels.

κ(PC 3741)-cDNA was purified by back-hybridization to κ(MOPC-41)-mRNA followed by S_1 nuclease digestion. Since κ3741 and κ41 share constant regions, but not variable regions, only the constant region of κ3741-cDNA should be protected from S_1 digestion. Indeed, when the κ-cDNA was sized as described above, only one band was seen and calculated to be about 620 nucleotides in length (data not shown). This corresponds to the 3′-untranslated region plus the constant region of κ-mRNA (about half the total 1200 bases in κ-mRNA). The κ-cDNA hybridized with κ41-mRNA with monophasic kinetics and reached a plateau of 100% in less than two orders of magnitude of R_0t units (B. Arp & R. Near, unpublished experiments).

Hybridization of α-cDNA with Whole-Cell and Cytoplasmic RNAs. Figure 5A shows the hybridization of α-cDNA with thymus whole-cell RNA to about 100%. Such hybridization was not observed with yeast or 66.2 and NP-2 myeloma whole-cell RNAs (Figures 5A and 5B). Therefore, the α-RNA sequences are not present in all cells. Spleen contains 1.2–3.5 times the concentration of α-RNA sequences of thymus (Table I, experiments 4 and 5) while the spleen/thymus ratio of κ-RNA sequences is about 2. The ratio of α/κ RNA sequences is 0.49 and 0.84 for thymus and spleen, respectively. The higher production of κ-RNA over α-RNA is expected in the spleen since about 95% of serum Ig contains

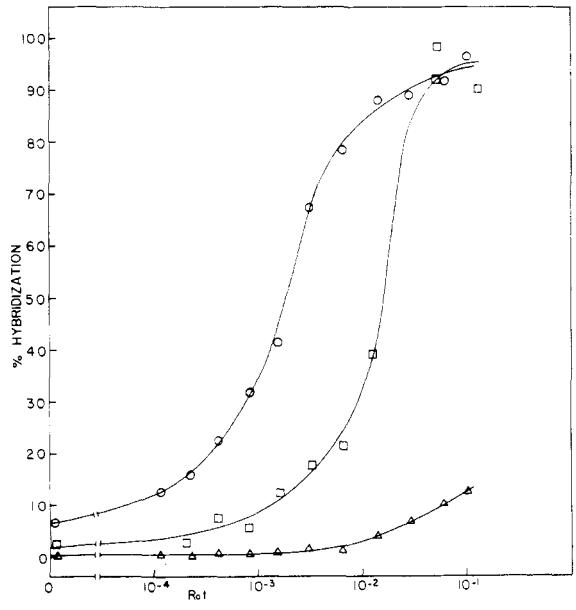

FIGURE 4: Characterization of α-cDNA by hybridization kinetics. α-cDNA was hybridized to formamide gel purified α(HOPC 2020)-mRNA (○—○); λ(HOPC 2020)-mRNA (□—□); and λ-(S178A)-mRNA (△—△). Hybridizations were done at mRNA excess as described in the text.

κ chains (Nisonoff et al., 1975) and 86% of all spleen cells are κ-RNA positive as judged by in situ hybridization with a κ-cDNA probe (Storb, 1978). The α-RNA sequences are also

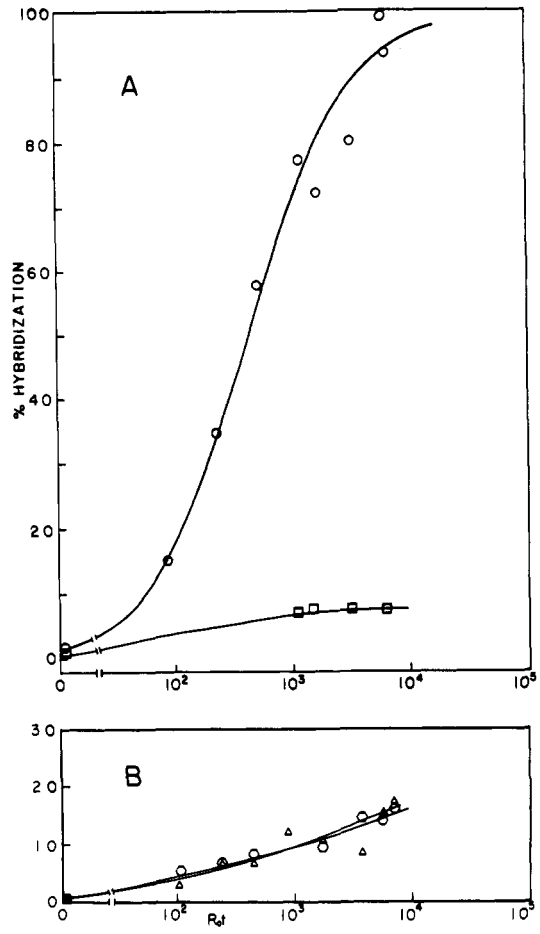

FIGURE 5: Hybridization kinetics of α-cDNA with whole cell RNAs. (A) α-cDNA was hybridized to thymus whole cell RNA (O—O) and to yeast RNA (□—□) (yeast RNA was a gift from Dr. R. Palmiter). (B) α-cDNA hybridized to NP-2 (△—△) and 66.2 (○–○) myeloma whole cell RNAs.

present in the thymus cytoplasm, although at one-third the concentration in whole-cell RNA (Table I, experiment 6). Further, there are about 3.8 times more κ- than α-RNA sequences in thymus cytoplasm (Table I, experiments 6 and 7). Assuming (1) a $R_0t/2$ for thymus α-RNA of 300, (2) that a pure α-mRNA yields a $R_0t/2$ of 1.8×10^{-3}, (3) that the average thymocyte contains 6 pg of RNA (Storb, unpublished results), and (4) that α-mRNA is 1900 nucleotides long, we calculate about 35 molecules α-RNA per thymocyte (κ-RNA represents about 75 molecules).

Thermal Denaturation of Hybrids. Figure 6 displays the melting profiles of α-cDNA hybrids with α-mRNA and with thymus whole-cell RNA, and κ-cDNA hybrids with thymus whole-cell RNA. The homologous reaction of α-cDNA to α-mRNA shows the sharpest melting transition with a T_m of 93.5 °C. The α-cDNA to thymus and κ-cDNA to thymus RNA hybrids show similar transition curves, both having a T_m of 91.0 °C. This indicates that in thymus RNA the α-RNA sequences are as well matched to α-cDNA as are κ-RNA sequences to κ-cDNA. The 2.5 °C difference between thymus α-RNA and α-mRNA hybrids with α-cDNA may not be significant since it only implies about 3% mismatching (Britten et al., 1974).

Discussion

The search for Ig associated with thymocytes has been going on for years without a definitive answer (Warner, 1974; Greaves, 1975). Ig reported to be associated with T cells

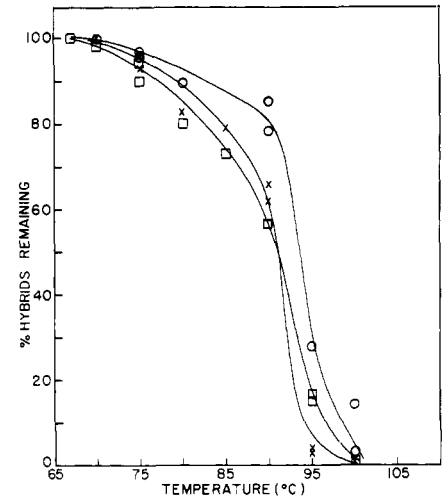

FIGURE 6: Thermal denaturation of α-cDNA and of κ-cDNA hybrids. κ- and α-cDNA were hybridized to a R_0t of 2800 with thymus whole cell RNA. α-cDNA was hybridized with α-mRNA to 0.4 R_0t unit. Melting of these hybrids is described in the text. α-cDNA and thymus RNA (□—□); κ-cDNA and thymus RNA (X—X); α-cDNA and α-mRNA (O—O).

appears to have different properties from B-cell Ig such as different solubility in detergent or slightly different sizes of Ig heavy chains (Cone & Brown, 1976). This is consistent with different antigen-binding properties of T and B cells (Hämmerling & McDevitt, 1974a,b). When Ig heavy chain was claimed to be present on T cells, it was most often found to be of the μ isotope (Warner, 1974; Marchalonis, 1975). Indeed, recent evidence with an unusual chicken anti-μ-chain antiserum has strongly indicated some part of μ chain to be present on T cells (Hämmerling, U., et al., 1976a,b). A similar chicken anti-κ-chain antiserum has also indicated κ chain to be present on T cells (Szenberg et al., 1977). It has been suggested, however, that such avian antisera are detecting variable region determinants and that the constant region of T-cell Ig is a yet uncharacterized IgT (Cone, 1977).

In agreement with the above are the findings of idiotypic determinants on T cells which are apparently identical with idiotypes found on B cells and serum Ig (Black et al., 1976; Binz & Wigzell, 1975a,b). The idiotypes examined were determinants found within the variable region of Ig heavy chain (Krawinkel et al., 1977) and were genetically linked to Ig heavy chain constant region markers (Hämmerling et al., 1976; Binz & Wigzell, 1976) but generally not histocompatibility markers. These idiotypic-positive molecules were reported to be composed of heavy-chain-like dimers of two 70 000 dalton chains (Binz & Wigzell, 1975c; Cone, 1977).

The lack of detection of Ig class specific determinants in the presence of idiotypic determinants supports the hypothesis of conventional v regions associated with IgT type c regions in T cells. However, it is possible that, for functional reasons, c regions but not v regions are altered in T cells by post-translational modifications, such as glycosylation. Such altered molecules may or may not react with antisera directed against c regions of B-cell immunoglobulins depending on the preparation and origin of the antiserum.

Of the heavy chain classes besides μ, α has also been reported to be synthesized by T cells (Moroz & Lahat, 1974). However, a later publication argues that this result is due to contamination of thymocytes with α-secreting plasma cells (Liswoska-Bernstein & Vassali, 1974).

We have used α-cDNA to search for α-RNA sequences in thymus T cells. The α-cDNA probe does not hybridize with λ-mRNA prepared from S178A myelomas. Since HOPC

2020, the source of our α-mRNA, produces both α and λ chains, our method for purification of α-mRNA seems to eliminate λ-mRNA aggregates. This is supported by the lack of λ chain found in the translation products of α-mRNA. The range of sizes of α-cDNA molecules is not continuous but shows several peaks of sizes (Figure 2). This result has also been reported for globin mRNA, chorion mRNA, and ovalbumin mRNA transcribed into cDNA (Efstratiadis et al., 1975). These incomplete, discrete peaks may result from the secondary structure of the mRNA (Lee & Hung, 1977) or possibly from certain base sequences affecting the reverse transcriptase. It is not likely that these peaks resulted from degraded α-mRNA used as incomplete templates, since the mRNA was excised as a sharp band of full-sized α-mRNA in formamide gels (similar RNA preparations have been rerun in formamide gels and appear as one band of RNA (R. Near, unpublished experiments)).

Secondary structure of RNA can affect its migration in formamide gels (Spohr et al., 1976). Furthermore, we have only sized the α-mRNA on tube rather than slab gels. Therefore, we cannot accurately assign a size to the α-mRNA. We estimate the size at 17–18.5 S (about 1900 nucleotides), while others have measured it as 16.5 S or 1800 nucleotides (Marcu et al., 1978). We do not have an explanation for this discrepancy but have used the 1900 figure for our calculations. The α-mRNA was probably not contaminated with 18S ribosomal RNA since no 28S ribosomal RNA was visible in formamide gels (Figure 1) and the kinetics of hybridization indicate the α-mRNA to be 87% pure.

Translation of the α-mRNA in the mRNA-dependent reticulocyte lysate system (Pelham & Jackson, 1976) showed the translation and immunoprecipitation of two bands, 53 500 and 57 500 daltons (Figure 2B). The translation of α(HOPC 2020)-mRNA in the wheat germ system has been reported to give one product of 55 000 daltons (Marcu et al., 1978). An analogous phenomenon has been reported with A^+RNA of HPC-108 plasmacytoma, an α producer (Schmeckpeper et al., 1977). These authors found most myeloma A^+RNAs produced only one band of a size corresponding to the precursor protein in the reticulocyte lysate system; however, HPC-108 produced two bands on $NaDodSO_4$ gels of 52 500 and 56 000 daltons. Further, translation of HPC-108 in the wheat germ system produced only the 56 000 dalton α precursor (Schmeckpeper et al., 1974). There are other reports that α chain does have a precursor protein (Jilka & Pestka, 1977). It is possible, therefore, that the α(HOPC 2020)-mRNA translated a precursor and a mature α chain in the reticulocyte system. We cannot, however, eliminate the possibility that the 53 500 dalton product represents a premature termination of translation (although the reticulocyte system seems very adept at completing protein chains once initiated (Pelham & Jackson, 1976)). There are some minor products translated, possibly resulting from premature termination or some α-mRNA degradation during translation since all such bands are smaller than α chains (Figure 2C). It is unknown how secondary structure of mRNA affects its translation in vitro, but it is conceivable that premature termination could result.

We have found α-chain RNA sequences in thymocytes as judged by the hybridization of α-cDNA to thymus RNA (Figure 5A). Immunofluorescence indicated that less than 0.02% κ-chain-containing plasma cells contaminated the thymocytes (κ chain is present in at least 95% of serum Ig molecules and should be a good indicator of the number of plasma cells contaminating thymocytes). This figure has been confirmed with the finding of only 0.015% of thymocytes showing high degrees of labeling by in situ hybridization using κ-cDNA (Storb, 1978). Therefore, it is unlikely that plasma cell contamination is responsible for the α-cDNA hybridization. Further, the finding of lower concentration of α-RNA sequences in cytoplasmic thymus RNA would seem inconsistent with plasma cell contamination since the plasma cell cytoplasm is very rich in endoplasmic reticulum devoted to Ig synthesis.

The α-RNA sequences in the thymus are well matched with α-mRNA sequences as shown by thermal denaturation curves of hybrids. However, these hybrids most likely represent the untranslated 3′ end of α-mRNA with little of the constant region. Hybrids representing the constant region may or may not have been included depending upon the amount of constant region present in the α-cDNA probe (anywhere from 2 to 22%; see Results). Nevertheless, the untranslated region is probably uniquely related to its translated region; for example, α-cDNA does not cross-react with either μ or γ Ig–mRNAs in hybridization assays (Marcu et al., 1978).

It appears that in both thymus and spleen there are more κ-RNA than α-RNA sequences (Table I). This could be on a single-cell basis or it could indicate the presence of α-RNA in only certain cells. Although there are more κ- than α-RNA sequences in spleen, the ratio of α- to κ-RNA is 0.84 which is much higher than expected (the α H chain is present in only a few percent of secreted Ig molecules (Sussdorf & McCann, 1975)). This may indicate that the α-RNA is translationally not as efficient as κ-RNA or that it is not transported into the cytoplasm as efficiently as κ-RNA.

We have found the presence of approximately 35 molecules of α-RNA per thymocyte. The cytoplasm of thymocytes contains these sequences at about one-third their concentration in the whole cell. This would be consistent with certain factors such as nuclear processing controlling the rate of transport of α-RNA from nucleus to cytoplasm. Nuclear processing has already been demonstrated for κ-RNA in myeloma cells (Gilmore-Hebert & Wall, 1978).

One should consider here whether there is any functional α-mRNA in the thymus cells. Approximately 80 to 90% of the total RNA of a thymus cell is present in the cytoplasm (Near & Storb, unpublished results). This and the fact that the concentration of RNA sequences in the cytoplasm was about one-third their concentration in the whole cell indicate that there are about 10 α-RNA molecules in the cytoplasm of the average thymus cell. It is unlikely that these α-RNA molecules were derived from contamination by nuclear sequences, because we did not observe any nuclear lysis—one-third of the nuclei would have had to be lysed to arrive at the observed quantity of α RNA in the cytoplasm. Also, in other experiments where the same methods of preparation of nuclei and cytoplasm were used, nucleus-restricted κ-RNA molecules were not found in the cytoplasm of a mastocytoma and a leukemia cell line (Storb et al., 1977). The presence of only 10 α-RNA molecules per average cell cytoplasm is probably sufficient for a molecule whose putative protein product functions as a receptor (Cuatrecasas, 1974). Further, the amount of amplification by translation may be a significant factor. The figure of 35 molecules per cell assumes that all thymus cells contain a few α-RNA molecules. It is quite possible that there are subpopulations of thymus cells which have much more or no such molecules. We are investigating such possibilities using in situ hybridization techniques (Storb, 1978). The levels of α-RNA found in thymus cells do not seem to be due to transcriptional "leakiness" occurring in all cells

since at least two myeloma cell types tested were devoid of α-RNA (Figure 5B). This suggests that the presence of α-RNA in T cells is of functional significance. Furthermore, we have found that thymus cells synthesize heavy chains in short-term culture which comigrate with myeloma α chains and which can be immunoprecipitated by anti-α antibodies (Putnam, Clagett, & Storb, unpublished results). However, we do not have any evidence at this time that these α-polypeptide sequences are inserted into the plasma membrane and used as functional receptors.

Added in Proof

Preliminary experiments have demonstrated that α-cDNA hybridizes to spleen cell cytoplasmic RNA at a $R_0t/2$ of about 950 (indicating that α-RNA is about one-fifth as concentrated in spleen cytoplasm as in spleen whole cells), while κ-cDNA hybridizes to spleen cytoplasm at a $R_0t/2$ similar to that of spleen whole cells (Storb and Near, unpublished results). Therefore, the behavior of α-RNA is similar in both spleen and thymus. While the behavior of α-RNA is different from κ-RNA, κ-RNA behaves similarly in thymocytes and spleen cells.

Acknowledgments

We gratefully acknowledge the help of Ben Arp in reviewing this manuscript, preparing κ-cDNA, and supplying helpful discussions. We thank Drs. James Clagett and D. Putnam for performing the immunofluorescence assays. Also Dr. Martin Weigert has kindly supplied the HOPC 2020, PC 3741, and PC 2880 myelomas. AMV reverse transcriptase was provided generously by Drs. J. Beard and M. Chirigos of the Office of Program Resources and Logistics, Viral Cancer Program, National Institutes of Health. Dr. R. Wilson kindly provided advice in setting up formamide gels.

References

Adamson, S. D., Herbert, E., & Kemp, S. F. (1969) *J. Mol. Biol. 42*, 247.

Ashman, R. F. (1973) *Immunology 26*, 539.

Bantle, J. A., Maxwell, I. H., & Hahn, W. E. (1976) *Anal. Biochem. 72*, 413.

Basten, A., Miller, J. F. A. P., Warner, N. L., & Pye, J. (1971) *Nature (London), New Biol. 231*, 104.

Binz, H., & Wigzell, H. (1975a) *J. Exp. Med. 142*, 197.

Binz, H., & Wigzell, H. (1975b) *J. Exp. Med. 142*, 1218.

Binz, H., & Wigzell, H. (1975c) *Scand. J. Immunol. 4*, 591.

Binz, H., & Wigzell, H. (1976) *Nature (London) 264*, 639.

Black, S. J., Hämmerling, G. J., Berek, C., Rajewsky, K., & Eichmann, K. (1976) *J. Exp. Med. 143*, 846.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83.

Britten, R. J., Graham, D. E., & Neufeld, B. R. (1974) *Methods Enzymol. 29*, 363.

Burton, K. (1956) *Biochem. J. 62*, 315.

Cantor, H., & Asofsky, R. (1970) *J. Exp. Med. 131*, 235.

Clagett, J. A., & Weigle, W. O. (1974) *J. Exp. Med. 139*, 643.

Cone, R. E. (1977) *Prog. Immunol. 3*, 47.

Cone, R. E., & Marchalonis, J. J. (1974) *Biochem. J. 140*, 345.

Cone, R. E., & Brown, W. C. (1976) *Immunochemistry 13*, 571.

Cuatrecasas, P. (1974) *Annu. Rev. Biochem. 43*, 169.

Efstratiadis, A., Maniatis, T., Kafutos, F. C., Jeffrey, A., & Vournakis, J. N. (1975) *Cell 4*, 367.

Gershon, R. K. (1973) *Contemp. Top. Immunobiol. 3*, 1.

Gilmore-Hebert, M., & Wall, R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 342.

Greaves, M. (1975) *Nature (London) 256*, 92.

Hämmerling, G. J., & McDevitt, H. O. (1974a) *J. Immunol. 112*, 1726.

Hämmerling, G. J., & McDevitt, H. O. (1974b) *J. Immunol. 112*, 1734.

Hämmerling, G. J., Black, S. J., Berek, C., Eichmann, K., & Rajewsky, K. (1976) *J. Exp. Med. 143*, 861.

Hämmerling, U., Mack, C., & Pickel, H. G. (1976a) *Immunochemistry 13*, 525.

Hämmerling, U., Pickel, H. G., Mack, C., & Masters, D. (1976b) *Immunochemistry 13*, 533.

Hilz, H., Weigers, U., & Adamietz, P. (1975) *Eur. J. Biochem. 56*, 103.

Honjo, T., & Kataoka, T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2140.

Jilka, R. L., & Pestka, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5692.

Katz, D., & Benacerraf, B. (1972) *Adv. Immunol. 15*, 2.

Kessler, S. W. (1975) *J. Immunol. 115*, 1617.

Kessler, S. W. (1976) *J. Immunol. 117*, 1482.

Krawinkel, U., Cramer, M., Mage, R. G., Kelos, A. S., & Rajewsky, K. (1977) *J. Exp. Med. 146*, 792.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335.

Lee, S. G., & Hung, P. P. (1977) *Nature (London) 270*, 366.

Lesley, J. F., Kettman, J. R., & Dutton, R. W. (1971) *J. Exp. Med. 134*, 618.

Lisowska-Bernstein, B., & Vassalli, P. (1974) *Biochem. Biophys. Res. Commun. 61*, 142.

Maniatis, T., Jeffrey, A., & van deSande, H. (1975) *Biochemistry 14*, 3787.

Marchalonis, J. J. (1975) *Science 190*, 20.

Marchalonis, J. J., Cone, R. E., & Atwell, J. L. (1972) *J. Exp. Med. 135*, 956.

Marcu, K. B., Valbuena, O., & Perry, R. P. (1978) *Biochemistry 17*, 1723.

Moroz, C., & Lahat, N. (1974) *Cell. Immunol. 13*, 397.

Nisonoff, A., Hopper, J. E., & Spring, S. B. (1975) in *The Antibody Molecule* (Dixon, F. J., & Kunkel, H. G., Eds.) p 5, Academic Press, New York.

Ono, M., Kondo, T., Kawakami, M., & Honjo, T. (1977) *J. Biochem. (Tokyo) 81*, 949.

Palmiter, R. D. (1973) *J. Biol. Chem. 246*, 7407.

Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247.

Putnam, D., Storb, U., & Clagett, J. (1977) *ICN-UCLA Symp. Mol. Cell. Biol. 6*, 71.

Schmeckpeper, B. J., Cory, S., & Adams, J. M. (1974) *Mol. Biol. Rep. 1*, 355.

Schmeckpeper, B. J., Cory, S., & Adams, J. M. (1977) *Biochim. Biophys. Acta 476*, 303.

Small, P. A., & Lamm, M. E. (1966) *Biochemistry 5*, 259.

Spohr, G., Mirault, M., Imaizumi, T., & Scherren, K. (1976) *Eur. J. Biochem. 62*, 313.

Storb, U. (1972) *J. Immunol. 108*, 755.

Storb, U. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2905.

Storb, U., & Marvin, S. (1976) *J. Immunol. 108*, 259.
Storb, U., Hager, L., Putnam, D., Buck, L., Farin, F., & Clagett, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2467.
Storb, U., Hager, L., Wilson, R., & Putnam, D. (1977) *Biochemistry 16*, 5432.
Sussdorf, D. H., & McCann, R. L. (1975) *Immunochemistry 12*, 607.
Swan, D., Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1967.
Szenberg, A., Marchalonis, J. J., & Warner, N. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2113.
Verma, I. M., Temple, G. F., Fan, H., & Baltimore, D. (1972) *Nature (London), New Biol. 235*, 163.
Vitetta, E. S., & Uhr, J. W. (1975) *Science 189*, 964.
Warner, N. L. (1974) *Adv. Immunol. 19*, 67.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Wilson, R. E., Walfield, A. W., Murakami, A., & Storb, U. (1978) *Biochemistry 17*, 3096.

Subunits of RNA Polymerase in Function and Structure. 7. Structure of Premature Core Enzyme†

Akira Ishihama,* Hiroji Aiba,‡ Tsunao Saitoh, and Sho Takahashi§

ABSTRACT: The structure of premature core enzyme, an obligatory intermediate in both in vivo and in vitro assembly of *Escherichia coli* DNA-dependent RNA polymerase, was compared with that of native core enzyme. Though this assembled but inactive form of core enzyme harbors the gross conformation similar to that of native enzyme, minor and presumably local differences exist, which were identified by near-ultraviolet circular dichroism spectra, tritium–hydrogen exchange rate, protease sensitivity, intersubunit cross-linking rate by bifunctional reagents, sedimentation behavior, and elution profile from phosphocellulose. Taken together these results indicate that the core enzyme subunits are loosely associated in the premature core. The temperature-dependent maturation is required for the core subunits to be tightly associated, leading to the formation of structurally stable and functionally active RNA polymerase.

The DNA-dependent RNA polymerase [ribonucleoside 5′-triphosphate:RNA nucleotidyltransferase (EC 2.7.7.6)] is the key enzyme for the transcription of genetic informations in *Escherichia coli*. The RNA polymerase[1] holoenzyme is a protomer of molecular weight about 500 000 and has subunit composition of $\alpha_2\beta\beta'\sigma$ with the molecular weight of each subunit being 38 500, 155 000, 165 000, and 87 000, respectively (Burgess, 1969; Berg & Chamberlin, 1970). The formation of the complex structure has been studied extensively in in vitro reconstitution systems and it has been revealed that the core enzyme subunits are assembled in a stepwise fashion under the following sequence:

$$2\alpha \rightarrow \alpha_2 \xrightarrow{\beta} \alpha_2\beta \xrightarrow{\beta'} \alpha_2\beta\beta' \text{ (premature core)} \rightarrow \text{E (active core)}$$

(Ishihama & Ito, 1972; Ishihama et al., 1973; Palm et al., 1975; Saitoh & Ishihama, 1976). Evidences have been obtained which indicate that the pathway of core enzyme assembly in vivo is identical with that found in vitro (Ito et al., 1975; Taketo & Ishihama, 1976, 1977). One of the remarkable features of the assembly mechanism is the formation of an assembled but inactive form of core enzyme ("premature core"), which indicates that proper arrangement of the subunits is necessary for the RNA polymerase activity to be exposed. In the in vitro reconstitution system, the activation of premature core ("enzyme maturation"), the rate-limiting step in the assembly sequence, can be achieved in the three different ways, i.e., self-reactivation, σ subunit-promoted reactivation, and DNA-promoted reactivation, which require respectively strict but different conditions (Saitoh & Ishihama, 1976). It remains, however, to be determined which of the three pathways operates in vivo. Systematic comparison of the structure and function of premature, reactivated, and native core enzymes is one of the ways to answer this question.

The present report deals with one of our efforts on the subject and describes the conformational differences between premature and native core enzymes.

Materials and Methods

Chemicals. Unlabeled ribonucleoside 5′-triphosphates were purchased from P-L Biochemicals and Boehringer Mannheim GmbH (West Germany), and labeled nucleotides were from Schwarz/Mann. Recrystallized products of urea, sodium dodecyl sulfate ($NaDodSO_4$), acrylamide, and *N,N′*-methylenebis(acrylamide) were obtained from Wako Chemical, Japan. Diisopropyl phosphofluoridate (Dip-F) and phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) were obtained from Sigma Chemicals. Beef liver catalase (EC 1.11.1.6) and trypsin (EC 3.4.21.4) were obtained from Sigma Chemicals, while β-galactosidase is a product of Boehringer Mannheim GmbH, West Germany. T7 DNA was purified by phenol extraction from phage stocks isolated by CsCl centrifugation.

† From the Department of Biochemistry, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan. *Received August 17, 1978; revised manuscript received October 25, 1978.* This work was supported in part by grants from the Ministry of Education, Science and Culture, of Japan and the Asahi Press. Paper 6 in this series is Saitoh & Ishihama (1976).

‡ Radioisotopes Laboratory, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.
§ Department of Enzyme Chemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan.

[1] Abbreviations used: RNA polymerase, ribonucleoside 5′-triphosphate:RNA nucleotidyltransferase (DNA-dependent) (EC 2.7.7.6); $NaDodSO_4$, sodium dodecyl sulfate; Dip-F, diisopropyl phosphofluoridate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; CD, circular dichroism; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

0006-2960/79/0418-0972$01.00/0