# Immunoglobulin $\mu$- and $\gamma$-Ribonucleic Acid Sequences in Thymocytes and Splenocytes from Normal and Hyperimmune Mice†

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ABSTRACT: The immunoglobulin heavy-chain ribonucleic acid (RNA) repertoire of mouse thymocytes was examined. Previously, this laboratory reported immunoglobulin $\alpha$-chain RNA sequences in mouse thymocytes [Near, R. I., & Storb, U. (1979) *Biochemistry 18*, 964]. We have extended these studies to encompass $\mu$, $\gamma_{2b}$, and $\gamma_1$ heavy-chain RNA sequences. $\mu$-, $\gamma_{2b}$-, and $\gamma_1$-messenger RNAs (mRNAs) were purified from myelomas to 45, 22, and 54% purity, respectively. Each of these mRNAs faithfully translated into the appropriate immunoprecipitable protein in a reticulocyte lysate translation system. The $\gamma_1$-mRNA translated into two major immunoprecipitable products of about 52 500 and 51 000 daltons while $\mu$- and $\gamma_{2b}$-mRNAs yielded only a single major protein. Complementary deoxyribonucleic acids (cDNAs) prepared from the mRNAs were used as hybridization probes and revealed the presence of about 70 $\mu$-RNA sequences per average thymocyte as determined by hybridization kinetics, while $\gamma_1$ and $\gamma_{2b}$ sequences were at the limits of detection. The $\mu$-RNA sequences are present in the cytoplasm and are >50% polyadenylated. Upon hyperimmunization of mice with sheep red blood cells, $\gamma_1$-RNA in splenocytes increased by about 100-fold while only slightly increasing in thymocytes. $\mu$ and $\gamma_{2b}$ increased 2–3-fold in splenocytes and only slightly in thymocytes. The results argue against RNA sequences appearing in thymocytes due to contamination with peripheral plasma cells. The results involving $\mu$- and $\gamma_{2b}$-RNAs were confirmed with cloned cDNA probes. Thymocyte RNA analyzed by Northern blots displayed bands of the same size as those in splenocyte RNA or in purified mRNA when hybridized to $\mu$, $\gamma_{2b}$, and $\alpha$ cloned probes. Also, $\kappa$ light-chain RNAs of the same size were found in spleen and thymus by using a cloned $\kappa$-DNA probe. The results are consistent with the thymus containing $\mu$-, $\alpha$-, and $\kappa$- and small amounts of $\gamma_1$- or $\gamma_{2b}$-RNAs coding for heavy- and light-chain-like proteins which may play a role in T-cell function.

Thymus-derived lymphocytes of the immune system are able to specifically recognize antigenic determinants via an antigenic receptor (Raff, 1970; Basten et al., 1971). Although these receptors are of great interest to immunologists and cell biologists, their molecular composition currently remains undefined. There is, however, accumulating evidence that the T-cell receptor contains immunoglobulin (Ig)[1] V-region sequences genetically linked to Ig heavy-chain allotypes (Rubin et al., 1979; Black et al., 1976; Binz & Wigzell, 1976a). The presence of the Ig C region in these molecules remains controversial when examined with immunochemical techniques (Warner, 1974; Marchalonis, 1975; Vitetta & Uhr, 1975). We have previously found that thymocytes metabolically labeled with [$^{35}$S]methionine produce immunoprecipitable $\kappa$ light chains associated with heavy-chain-like molecules (Putnam et al., 1980). Under the conditions used, it is unlikely that these products were due to contaminating B cells or plasma cells.

The use of nucleic acid technology avoids many of the difficulties of immunochemical techniques [for a discussion, see Storb et al. (1980)]. We have therefore used such techniques and have demonstrated the presence of $\kappa$-RNA (Storb et al., 1976, 1977; Storb, 1978) and $\alpha$-RNA sequences (Near & Storb, 1979) in mouse thymocytes. The $\alpha$ sequences are present at about 40% the concentration of $\kappa$-RNA sequences in thymocytes.

Since the $\mu$ chain has been the most commonly reported Ig on T cells (Warner, 1974; Marchalonis, 1975; Hämmerling et al., 1976a,b), we have now examined thymocytes for $\mu$-RNA sequences.[2] The use of cloned $\mu$-DNA hybridization probes in these studies has eliminated any question of probe purity. We find approximately 70–80 copies of $\mu$-RNA per cell. In contrast to $\mu$-RNA, we find few $\gamma$-RNA sequences in thymocytes by using $\gamma_1$-cDNA and $\gamma_{2b}$ cloned hybridization probes. Apparently, not every immunoglobulin isotype is expressed to the same degree in thymocytes.

## Experimental Procedures

*Mouse Strains and Myelomas.* PC 3741 ($\mu\kappa$) and PC 2880 ($\gamma_{2b}\kappa$) were kindly donated by Dr. M. Weigert and passaged in (BALB/c × NZB)F1 mice by subcutaneous injection. MOPC-21 ($\gamma_1\kappa$) and MOPC-41 ($\kappa$) were obtained from Dr. M. Potter through Litton Bionetics and passaged in BALB/c mice.

*Splenocyte and Thymocyte Preparations.* These techniques have been previously described in detail (Near & Storb, 1979). As before, thymocytes were checked for B-cell and plasma cell contamination by immunofluorescence (Near & Storb, 1979; Putnam et al., 1980). There was only a small contamination with 0.02% plasma cells and 0.3% surface-positive B cells on

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; cDNA, complementary DNA; ssDNA, single-stranded DNA prepared from cloned cDNA; $R_0t$, product of the concentration of the nucleotide sequence of RNA and the time of incubation (mol of nucleotides·s/L); $R_0t_{1/2}$, $R_0t$ value at 50% plateau hybridization; $T_m$, temperature of 50% thermal denaturation of hybrids; Ig, immunoglobulin; $A^+$RNA, polyadenylated RNA; kb, kilobases; SRBC, sheep red blood cells; DBM, diazobenzyloxymethyl; C region, constant region; H, heavy chain; V region, variable region; PPO, 2,5-diphenyloxazole; $Me_2SO$, dimethyl sulfoxide.

[2] Preliminary data indicating the presence of $\mu$-RNA and a low level of $\gamma_1$-RNA in thymocytes were presented in a poster session at the 18th Midwinter Conference of Immunologists (Jan 1979) and in Storb et al. (1980). More comprehensive data with cloned probes were presented at the 19th Midwinter Conference (1980).

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the average. Immune splenocytes and thymocytes were prepared from BALB/c mice injected intraperitoneally with 0.2 mL of a 30% suspension of washed sheep red blood cells (SRBC) on day 0 and day 6 (Church et al., 1968). Mice were sacrificed on day 14 and spleens and thymuses removed.

*Whole-Cell RNA Preparations.* These procedures have also been previously described (Near & Storb, 1979). DNase I (Worthington) was passed over agarose-coupled (aminophenyl)phosphoryluridine 2′(3′)-phosphate (Maxwell et al., 1977) to remove any RNase activity. This DNase was used to destroy any residual DNA from RNA preparations. RNA treated with such DNase remains undegraded as assayed in glyoxal agarose gels (R. I. Near, unpublished experiments).

*Purification of μ(3741), $\gamma_1$(21), and $\gamma_{2b}$(2880) mRNAs.* These mRNAs were prepared as described for α-mRNA (Near & Storb, 1979) with the additional step of a gradient fractionation (Marcu et al., 1978) before the $A^+$RNA was applied to formamide gels. This additional step is needed since κ-mRNA aggregates may contaminate heavy-chain mRNA to a small extent (R. I. Near, unpublished experiments).

*In Vitro Translations.* RNA samples were translated in a reticulocyte lysate system made mRNA dependent with micrococcal nuclease (Pelham & Jackson, 1976). [$^{35}$S]-Methionine (Amersham) at 250 μCi/mL was the source of label for all reactions. The exact translation conditions and the processing of the translated proteins have been described in detail (Near & Storb, 1979). Specific proteins were immunoprecipitated by using a staphylococcal protein A–antibody system (Kessler, 1976) with antisera specific for the $\gamma_1$, $\gamma_2$, or μ heavy chains (Meloy and Bionetics) as previously reported (Near & Storb, 1979).

*NaDodSO$_4$–Polyacrylamide Gel Electrophoresis.* Translation products, immunoprecipitates, and cold myeloma protein markers were run on 10% acrylamide–0.3% bis(acrylamide) discontinuous NaDodSO$_4$ slab gels (Laemmli, 1970). The gels were stained with Coomassie blue, Me$_2$SO–PPO impregnated for fluorography (Bonner & Laskey, 1974) and exposed to preflashed Kodak X-Omat R X-ray film at −70 °C (Laskey & Mills, 1975).

*Hybridization Probes.* Preparation of synthetic [$^3$H]cDNA probes with avian myeloblastosis reverse transcriptase and its further purification by back-hybridization to its template mRNA were as previously described (Near & Storb, 1979).

cDNA clones for preparation of cloned single-stranded hybridization probes (ssDNA) were obtained from A. Bothwell (pABμ-1) and R. P. Perry [P$\gamma_{2b}$(11)$^7$]. The μ insert of pABμ-1 is approximately 800 nucleotides representing the 3′ end of the μ-mRNA placed into the *Pst*I site of pBR322 by G-C tailing. The μ insert may be easily removed via *Pst*I cleavage followed by 5–20% sucrose-gradient centrifugation containing 1 M NaCl, 1 mM EDTA, and 20 mM Tris, pH 7.5 A 50-μg sample of DNA was fractionated on a 5-mL gradient spun in a Beckman SW 50.1 rotor at 48 000 rpm for 5.5 h. The $\gamma_{2b}$ insert of p$\gamma_{2b}$(11)$^7$ represents the terminal 1.1 kb of the 3′ end of $\gamma_{2b}$-mRNA placed into the *Eco*RI site of pMB9 via A-T tailing and can be cleaved from the plasmid by using S1 nuclease under partially denaturing conditions (Hofstetter et al., 1976). Gradient centrifugation was used to isolate this $\gamma_{2b}$ insert as well.

Single-stranded cloned hybridization probes were prepared from the isolated inserts as described by Young et al. (1978) with the following minor modifications. The double-stranded cDNA inserts were nick translated as before (Wilson et al., 1979) except that 1500 pmol of both radioactive [$^3$H]dCTP at 60 Ci/mmol and [$^3$H]dTTP at 100 Ci/mmol (New England Nuclear) and cold precursors were used and that DNase (Worthington) was used at 125 ng/mL. Tritium label was preferred since it provided a stable probe for several solution hybridization experiments. The nick translation resulted in a specific activity of 10–20 cpm/pg. The nick-translated insert was boiled 3 min and then annealed in a 100-μL capillary with a 10-fold sequence excess of homologous mRNA (which need not be a pure preparation) in 50% formamide, 0.5 M NaCl, 50 mM *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.0, 0.5 mM EDTA, and 10 μg/mL *B. subtilis* RNA at 67 °C. After an overnight incubation, the capillary was quick-frozen in dry ice–acetone, and the contents were diluted into 1 mL of S1 buffer (30 mM sodium acetate, 0.6 mM ZnSO$_4$, and 50 mM NaCl, pH 4.5, containing 30 μg/mL *B. subtilis* RNA) and treated for 1 h at 37 °C with 188 units of S1 nuclease (Sigma). Following phenol–chloroform (1:1) extraction and reduction to a volume less than 0.5 mL with 2-butanol extractions (Stafford & Bieber, 1975), the solution was fractionated over Sephadex G-75 in 10 mM Tris, 10 mM NaCl, and 1 mM EDTA, pH 7.5. The RNA–DNA hybrids in the void volume fraction were treated with 0.3 N NaOH at 65 °C for 12 min. After neutralization, the ssDNA was ethanol precipitated with *B. subtilis* RNA as carrier.

In addition to the above plasmids, two other plasmids were used as hybridization probes for Northern blots (see below). p167κRI was obtained from P. Early (Joho et al., 1980), and a $C_\kappa$ fragment was isolated as previously described (Wilson et al., 1979). pα8 was obtained from G. Sonenshein and A. Bothwell and contains about 700 nucleotides from the 3′ end of α-mRNA inserted into the *Pst*I site of pBR322. The entire pα8 plasmid was used for Northern blots.

All recombinant plasmids were maintained in compliance with NIH guidelines on recombinant DNA.

*"Northern" Blots.* RNA was treated with 1 M glyoxal in 50% Me$_2$SO at 50 °C to achieve denaturation (McMaster & Carmichael, 1977). These RNA samples were electrophoresed and blotted by a procedure previously described (Storb et al., 1980) as suggested by Alwine (J. C. Alwine, unpublished experiments). Approximately 15 μg of RNA per lane was electrophoresed at 50 mA in 10 mM phosphate, pH 6.8. The gel was briefly alkaline treated, neutralized, and blotted against DBM paper (Alwine et al., 1977). Hybridization to the paper was the same as we have used for Southern blots by using $^{32}$P-labeled nick-translated plasmid DNA (Wilson et al., 1979). In general, splenic RNA was prepared by the guanidine method as opposed to proteinase K (Near & Storb, 1979) since the latter technique leaves splenic RNA slightly degraded. We have successfully reused blots, washing away hybridized probe by heating to 65 °C for 1 h in the presence of 99% formamide.

More recently, we have blotted the RNA onto nitrocellulose rather the DBM paper with a procedure described by Thomas (1980). Northern blot films were scanned with a Helena Laboratories Quick Scan densitometer. The relative areas occupied by hybridization bands were determined by a Quick-Quant II integrator.

## Results

*Purification of μ-, $\gamma_1$-, and $\gamma_{2b}$-mRNAs.* Each H-mRNA was characterized by hybridization kinetics with its homologous cDNA and by translation in the mRNA-dependent reticulocyte lysate system (Pelham & Jackson, 1976). The characterization of the H-mRNAs and cDNAs is summarized in Table I. The cDNA probes are significantly purer than their template mRNAs since the cDNAs were further purified by back-hybridization to their template mRNAs for a short $R_0t$ period (Storb et al., 1977). The cDNAs are of sufficient

Table I: Characterization of H-mRNAs and cDNAs

| mRNA | source | size[a] | $R_0t_{1/2}$[b] | % RNA purity[c] | cDNA size[d] | % C region in cDNA[e] |
|---|---|---|---|---|---|---|
| $\mu$ | PC 3741 | 2200 | $4.5 \times 10^{-3}$ | 45 | 30–1100 | 65 |
| $\gamma_1$ | MOPC 21 | 1900 | $3.2 \times 10^{-3}$ | 54 | 100–1300 | 80 |
| $\gamma_{2b}$ | PC 2880 | 1900 | $8.0 \times 10^{-3}$ | 22 | ND | ND |

[a] Size of the heavy-chain RNAs has been determined on formamide–polyacrylamide gels as described (Near& Storb, 1979) and is given in nucleotides. [b] The H-mRNA is hybridized to its homologous cDNA in RNA excess as described (Near & Storb, 1979). Note: none of the H-cDNAs hybridized with $\kappa$-RNA. [c] Purity is calculated by comparing the $R_0t_{1/2}$ value with the $R_0t_{1/2}$ value of a highly purified $\kappa$-RNA standard ($1.1 \times 10^{-3}$), adjusting for the different mRNA sizes. All hybridizations reached plateaus of over 80% hybridization. [c] cDNA sizes (nucleotides) have been determined on Tris–borate gels (Near & Storb, 1979; Maniatis et al., 1975). ND, not determined. [e] Determined from the average size of the cDNAs and knowing that the length of the 3′-untranslated region is about 100 nucleotides for $\gamma_1$ (Honjo et al., 1979) and about 150 for $\mu$ (Gough et al., 1980).

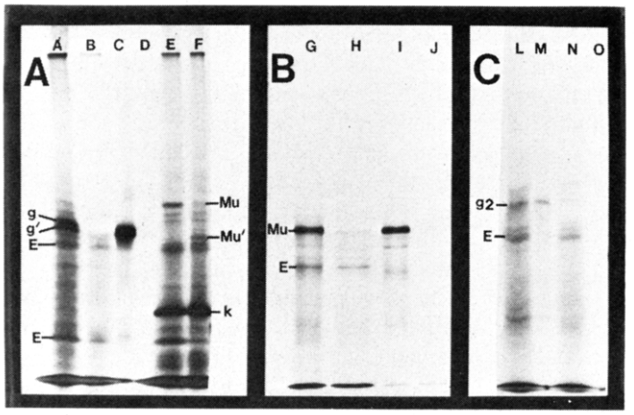


FIGURE 1: $NaDodSO_4$–polyacrylamide gels (10%) of reticulocyte lysate translations of H-mRNAs. (A) Fluoroautoradiograph of a gel containing $\gamma_1$-mRNA translation products and immunoprecipitates (lanes A–D) and $A^+$RNA from PC 3741 (lanes E and F). The lanes contained the following samples: (lane A) translation of $\gamma_1$-mRNA; (lane B) supernatant of the translation products following immunoprecipitation with anti-$\gamma_1$ antiserum; (lane C) immunoprecipitate with anti-$\gamma_1$ antiserum of $\gamma_1$-mRNA translation products; (lane D) control immunoprecipitate by using normal goat serum; (lane E) translation products of $A^+$RNA from PC 3741 during an early passage; (lane F) same as (E) except during a later tumor passage. (B) Autoradiograph of an $NaDodSO_4$ gel containing $\mu$-RNA translation products. The lanes contained the following samples of $\mu$-RNA translates: (lane G) whole translation products; (lane H) postimmunoprecipitate supernatant; (lane I) immunoprecipitate with anti-$\mu$ antiserum; (lane J) control immunoprecipitate. (C) Fluoroautoradiograph of an $NaDodSO_4$ gel containing $\gamma_{2b}$-mRNA translation products. The lanes contained the following samples: (lane L) whole translation products; (lane M) immunoprecipitate with anti-$\gamma_{2b}$; (lane N) postimmunoprecipitate supernatant; (lane O) control immunoprecipitate (g, g′, $\gamma_1$ chains; E, endogenous products of the translation system; $\kappa$, $\kappa$ chain; M$\mu$, $\mu$ chain; g2, $\gamma_{2b}$ chain; M$\mu'$, putative modified $\mu$ chain).

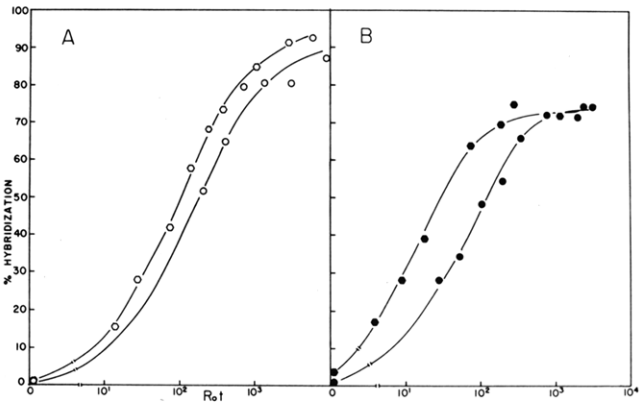


FIGURE 2: Hybridization kinetics of $\mu$-cDNA with whole-cell RNAs. (A) $\mu$-cDNA hybridized to thymocyte whole-cell RNAs from normal (○) or hyperimmune (○) mice. (B) $\mu$-cDNA hybridized to splenocyte whole-cell RNAs from normal (●) or hyperimmune (●) mice.

length such that most of the C region is represented with little or no V region. $\gamma_{2b}$-mRNA was only about 22% pure; however, a $\gamma_{2b}$-ssDNA probe was available. Although the purities of $\mu$- and $\gamma_1$-mRNAs are somewhat less than that of $\alpha$-mRNA (87%; Near & Storb, 1979), they are quite adequate for studies involving RNA-excess hybridization, considering the cDNAs are purer than the mRNAs. The cDNAs hybridized to their homologous mRNAs within two log ranges, and the hybridizations reached plateaus of >80% hybridization. The cDNAs do not hybridize to $\kappa$-mRNA, the other major mRNA produced by the myelomas used for H-mRNA purification. We estimate the cDNAs to be at least 50%, and probably 80–90%, pure.

Upon translation in a mRNA-dependent reticulocyte system, we found $\gamma_1$-mRNA translates into two equally intense immunoprecipitable bands, g and g′ (Figure 1A, lanes A and C), of approximately 52 500 and 51 000 daltons, respectively. Control precipitates show little or no material (lane D). $\mu$-mRNA translates into one immunoprecipitable band of about 67 000 daltons (Figure 1B, lanes G and I); however, the immunoprecipitate includes some smaller products which may result from breakdown or premature termination, and the control precipitate contains a small amount of material the size of the $\mu$ chain which has not been further analyzed. $\gamma_{2b}$-mRNA translates into one major immunoprecipitable band (Figure 1C, lanes L and M) and a minor $\kappa$-chain band, indicating only very little $\kappa$-mRNA contamination since $\kappa$-mRNA translation is about twice as efficient as $\gamma_{2b}$-mRNA translation (R. Near, unpublished experiments).

One of the major difficulties in the purification of $\mu$-mRNA involves the frequent loss of mature $\mu$-mRNA during in vivo tumor passage. An example of this is shown in Figure 1A (lanes E and F). $A^+$RNA from an early tumor passage clearly yields $\mu$-chain translation product; however, $A^+$RNA from a later passage (as little as two passages) produces considerably less $\mu$ chain but does produce a new polypeptide, $\mu'$ (lane F), of about 49 000 daltons. The similarity of $\mu'$ to the authentic $\mu$ chain, if any, has not yet been examined. Sucrose gradients of $A^+$RNA from the later-passage tumor show a drastic decrease in the $\mu$-mRNA peak (not shown), suggesting the lack of $\mu$ chain is due to the lack of the corresponding messenger, not a translational inhibition.

*Hybridization with Normal Splenocytes and Thymocytes.* RNA from single-cell suspensions of thymus or spleen cells from normal BALB/c mice was hybridized to the cDNA and cloned ssDNA probes (see below). Figure 2 shows the hybridization of $\mu$-cDNA to thymus (Figure 2A) and spleen (Figure 2B) whole-cell RNAs, reaching plateaus of about 90% and 75%, respectively. The $R_0t_{1/2}$ values indicate about 87 molecules of $\mu$-RNA per average thymocyte and 195 per average splenocyte (Table II).

Upon hybridization with the $\gamma_1$-cDNA probe, we found thymocytes to have levels of $\gamma_1$-RNA at the limits of detection

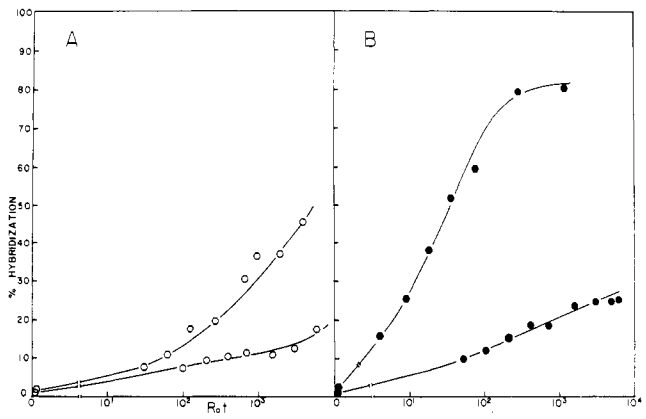


FIGURE 3: Hybridization kinetics of $\gamma_1$-cDNA with whole-cell RNAs. (A) $\gamma_1$-cDNA hybridized to thymocyte whole-cell RNAs from normal (○) or hyperimmune (○) mice. (B) $\gamma_1$-cDNA hybridized to splenocyte whole-cell RNAs from normal (●) or hyperimmune (●) mice.

of the hybridization assay (Figure 3A and Table II). Further, $\gamma_1$-RNA was almost undetectable in the normal splenocytes as well (Figure 3B and Table II). In view of the findings that adult splenocytes possess only about 0.5% cells positive for surface IgGl (Abney et al., 1978), these results are understandable (young mice, which we used, have still lower IgGl positive cells).

Normal thymocyte RNA also showed almost undetectable $\gamma_{2b}$-RNA sequences with either $\gamma_{2b}$-cDNA or $\gamma_{2b}$-ssDNA (Table II). In normal splenocytes, about 15 copies of $\gamma_{2b}$-RNA were found (Table II).

Thermal denaturation of the μ-cDNA–thymus RNA hybrids yields a $T_m$ of 91.5 °C (data not shown) with a relatively sharp transition. This is within 2 °C of cDNA–mRNA hybrids (Near & Storb, 1979) and suggests about 2–3% mismatching (Britten et al., 1974). We believe this degree of mismatching not to be experimentally significant. The μ-RNA sequences are found within the thymocyte cytoplasm (Table II) at about the same concentration as in thymocyte whole-cell RNA. Since most of the thymic RNA is present in the cytoplasm (R. I. Near and U. Storb, unpublished experiments), most of the μ-RNA is also cytoplasmic. Further, $A^+$RNA from thymocytes contains at least 50% of the μ-RNA in thymocytes as determined by hybridization kinetics with $A^+$RNA and the known yield of $A^+$RNA from whole-cell RNA (data not shown). Similarly, most of splenic μ-RNA is also cytoplasmic (Table II), and a significant portion is polyadenylated (Figure 6B).

*Hybridization to Immune Splenocytes and Thymocytes.* Since normal splenocytes contained only very low levels of $\gamma_1$- and $\gamma_{2b}$-RNAs in contrast to μ-RNA, we immunized mice with a complex antigen (SRBC) to develop a secondary immune response (Church et al., 1968). Similar procedures have been used by Honjo et al. (1977) to amplify $\gamma_1$-RNA sequences in splenocytes.

Figure 2 shows that immunization has a small effect upon μ-RNA in both thymocytes and splenocytes (also see Table II). Thymocyte RNA shows a slight increase (see Discussion) in $\gamma_1$-RNA while splenocytes display a dramatic 100-fold increase in $\gamma_1$-RNA upon immunization (Figure 3). $\gamma_{2b}$-RNA apparently does not undergo amplification to nearly the same extent as $\gamma_1$-RNA in immune splenocyte RNA, displaying only about a 2–3-fold increase as detected with the $\gamma_{2b}$-ssDNA probe (Table II).

*Hybridizations with Cloned Probes.* In order to eliminate any problems related to impure probes, we have used cloned single-stranded [$^3$H]cDNAs (ssDNA). Probes for both μ- and

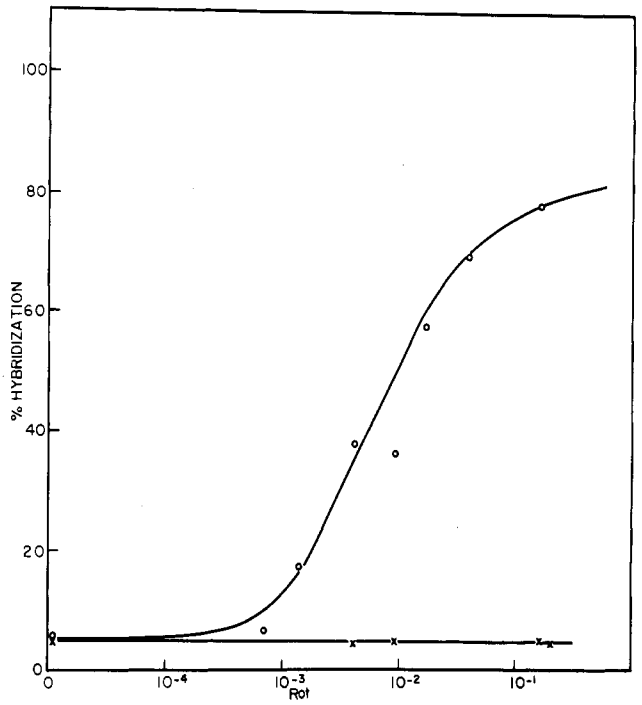


FIGURE 4: Characterization of the cloned μ-ssDNA hybridization probe. Cloned μ-ssDNA probe was hybridized to a crude (20% pure) μ-mRNA sample (○) or incubated alone (×).

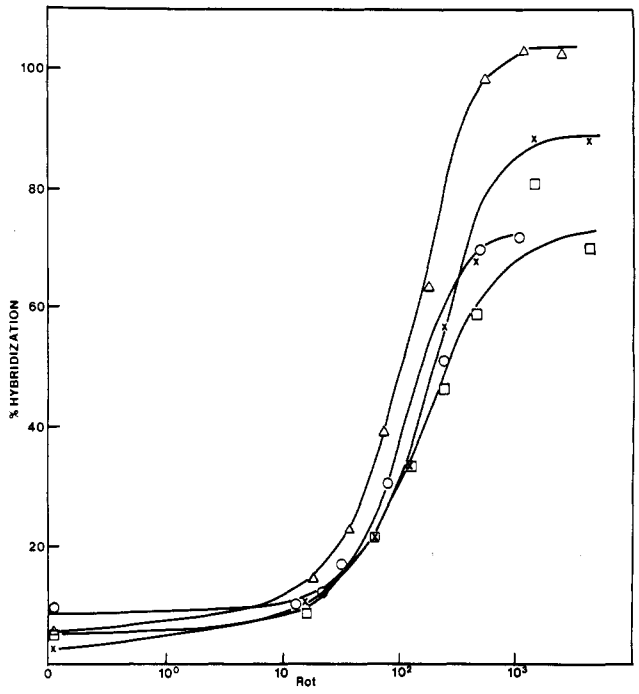


FIGURE 5: Hybridization kinetics of cloned μ-ssDNA with thymocyte RNAs. Cloned μ-ssDNA hybridized to thymocyte RNA from normal mice (□) or from hyperimmune mice (○, ×, and △).

$\gamma_{2b}$-RNAs have been reported. Figure 4 shows that the μ probe is single-stranded and can recognize μ-mRNA. The μ probe alone exhibits essentially no self-annealing over a period of about 24 h. This probe was then hybridized to splenocyte and thymocyte RNA preparations (Figure 5 and Table II). The results are comparable to the results with μ-cDNA and are reproducible with several different RNA preparations of immune thymus RNA (Figure 5 and Table II). Normal thymocytes have about 70 molecules of μ-RNA per average cell which increases only slightly or not at all upon immunization (66–91 molecules/cell). Immune splenocyte RNA shows 500 μ-RNA molecules/cell with μ-ssDNA, a result not

Table II: $\mu$ and $\gamma$ Probe Hybridizations to Cellular RNAs

| isotype of probe | probe sources: cDNA | probe sources: ssDNA | RNA source[a] | $R_0t_{1/2}$ | molecules/cell[b] |
|---|---|---|---|---|---|
| $\mu$ | + | | normal spleen | 54 | 195 |
| | + | | immune spleen[c] | 16.5 | 640 |
| | + | | normal thymus | 120 | 87 |
| | + | | immune thymus[c] | 80 | 130 |
| | | + | normal spleen | 60 | 175 |
| | | + | immune spleen[c] | 21 | 500 |
| | | + | normal thymus | 150 | 70 |
| | | + | immune thymus | 160 | 66 |
| | | + | immune thymus[c] | 115 | 91 |
| | | + | immune thymus | 122 | 86 |
| | + | | normal thymus cytoplasm | 120 | 70[d] |
| | | + | normal spleen cytoplasm | 48 | 175[d] |
| $\gamma_1$ | + | | normal spleen | >2000 | <5[e] |
| | + | | immune spleen[c] | 19 | 550 |
| | + | | normal thymus | >2000 | <5[e] |
| | + | | immune thymus[c] | ≥2000 | ≤5[e] |
| $\gamma_{2b}$ | | + | normal spleen | 680 | 15 |
| | | + | immune spleen | 325 | 34[e] |
| | + | | normal thymus | >2000 | <5[e] |
| | | + | immune thymus | 1600 | 7[e] |
| | | + | normal thymus | >2000 | <5[e] |

[a] All RNAs were prepared from single-cell suspensions by a proteinase K method (Near & Storb, 1979). [b] The number of molecules of an RNA sequence per average cell was calculated from the $R_0t_{1/2}$ value as described (Near & Storb, 1979). [c] Identical immune thymus or spleen RNA preparations. [d] Molecules per cell for cytoplasmic preparations were calculated on the basis of 80–90% of splenic or thymic RNA being cytoplasmic (R. I. Near, unpublished experiments). [e] $R_0t_{1/2}$ values for these hybridizations are approximate due to poorly defined plateaus.

significantly less than the 640 molecules/cell detected with $\mu$-cDNA, i.e., an increase of about 2.5-fold upon immunization. The $\gamma_{2b}$-ssDNA exhibits 12% self-annealing (not shown). Results with this probe have been discussed above and in normal thymocytes are comparable to results with $\gamma_{2b}$-cDNA (Table II).

*Northern Blots of Thymocyte and Splenocyte RNAs.* The size of Ig-RNA sequences in thymocytes compared to those in splenocytes was used as a criterion of authenticity. Thus, we completely denatured RNA with glyoxal (McMaster & Carmichael, 1977) and formed blots onto DBM paper or nitrocellulose after electrophoresis in 1.5% agarose gels (Alwine et al., 1977; Thomas, 1980). The same DBM blot was rehybridized to more than one probe (Figure 6B,D).

In each case, thymocyte RNA displays bands of identical size as splenocyte RNA (Figure 6) or respective H-mRNAs (not shown for $\alpha$- or $\mu$-mRNAs). Small amounts of $\gamma_{2b}$-RNA were found in normal thymocyte RNA (Figure 6C, lane a). Such small amounts do not necessarily conflict with the previous hybridization kinetics (see Discussion). The $\alpha$ bands in Figure 6D appear faint; however, this same blot had been rehybridized 4 times, $\alpha$ being the fourth. Each time a blot is rehybridized it loses intensity. For example, $\gamma_{2b}$ was the third probe hybridized to this blot with the result of no visible bands appearing (not shown). The thymocyte preparations contained significant amounts of $\kappa$-RNA, representing about one-fourth the amount of splenocyte $\kappa$-RNA as determined by scanning the radioautographs (Figure 6A, lane a compared to c; unfortunately, the splenocyte RNA was slightly degraded). The finding of $\kappa$-RNA by Northern blots supports previous liquid hybridization data (Near & Storb, 1979; Storb et al., 1976, 1977, 1980).

The $\mu$-RNA blot in normal thymocytes is diffuse with a band visible at 2.35 kb. The diffuseness is consistent with there being a shorter $\mu$-RNA of about 2.0 kb similar to that reported by others (Kemp et al., 1980b); however, we have not visualized a 3.0 kb $\mu$-RNA. No such diffuseness of bands was seen by using $\alpha$, $\kappa$, or $\gamma_{2b}$ probes, even when hybridizing to the same blot as well as the same thymus RNA samples. Both $\gamma_{2b}$- and $\alpha$-RNAs are approximately 1.9 kb in both spleen and thymus, while $\kappa$-RNA is 1.2 kb. Oligo(dT)–cellulose nonadherent RNA from PC 3741 ($\mu\kappa$) microsomes hybridizes to the $\mu$ probe, but in a band smaller than $\mu$-mRNA (Figure 6B, lanes a, d, g, and i), possibly due to loss of poly(A) tails. Neither $\alpha$ nor $\gamma_{2b}$ probes hybridized to this RNA (Figure 6D; not shown for the $\gamma_{2b}$ probe). This suggests that not all lymphoid cells produce all Ig RNAs. MOPC-41 $A^+$ microsomal RNA hybridizes to the $\mu$ probe. This myeloma does not produce $\mu$ polypeptide chains. It is possible that in MOPC-41 a nonfunctional $\mu$-RNA is produced and translated with a frameshift, in analogy to findings with nonfunctional $\kappa$-RNA (Walfield et al., 1980; A. Walfield and U. Storb, unpublished experiments). This has not yet been investigated.

In all cases, SRBC immunization of mice correlated with an increase of Ig-RNA band intensity in both thymocyte (Figure 6A,C, lane b; not shown for $\alpha$ or $\mu$ probes) and splenocyte RNA (not shown). This increase existed despite the finding that cytoplasmic Ig-positive cells as detected by immunofluorescence were 3-fold lower in this particular preparation of immune thymocytes. It is possible that hyperimmunization with SRBC stimulates T cells like a polyclonal activator.

## Discussion

In order to study whether thymocytes contain $\mu$-, $\gamma_{2b}$-, and $\gamma_1$-RNAs, we have purified the respective mRNAs from mouse myelomas and synthesized cDNA hybridization probes. Translation of these mRNAs leads to the production of one major immunoprecipitable band for $\mu$- and $\gamma_{2b}$-mRNAs and two bands for $\gamma_1$-mRNA (Figure 1). The two bands for $\gamma_1$-mRNA are reminiscent of what we found for a $\alpha$-mRNA (Near & Storb, 1979) and what has been reported before in both the wheat germ and the reticulocyte systems (Ono et al., 1977; Cowan & Milstein, 1973). The two bands could represent a precursor and a mature protein or a strong stop for premature termination of translation. There is another pos-

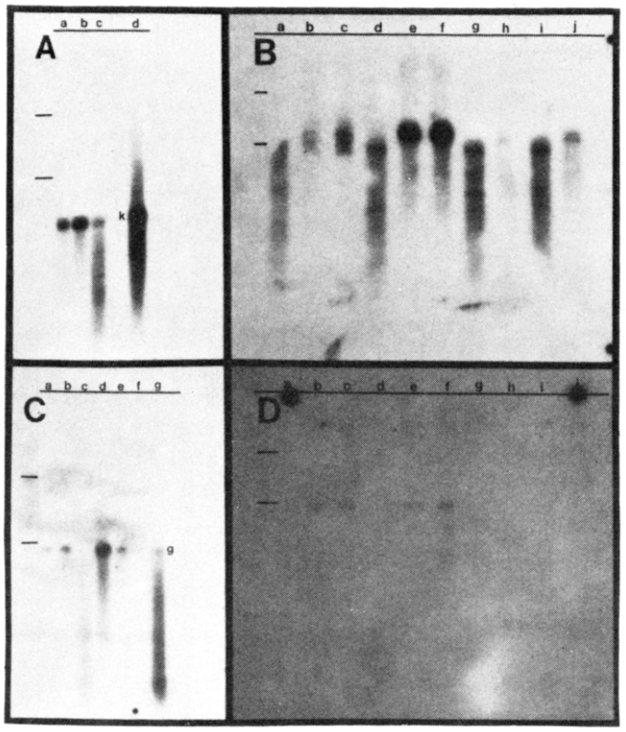


FIGURE 6: Northern blots of thymocyte and splenocyte RNAs. (A) Autoradiogram of hybridization to a nitrocellulose Northern blot with a $C_\kappa$ probe from p167. Lanes contained the following: (lane a) normal thymocyte $A^+$RNA (5 μg); (lane b) immune thymocyte $A^+$RNA (5 μg); (lane c) normal spleen $A^+$RNA (5 μg, partially degraded); (lane d) κ-mRNA (PC 3741: 30 ng). (B and D) Autoradiogram of hybridizations to a single DBM Northern blot. Lanes contained the following: (lanes a, d, g, and i) PC 3741 microsomal RNA nonadherent to oligo(dT)–cellulose (8.1 μg); (lanes b and c) $A^+$RNA from normal thymocytes (4 and 5.5 μg, respectively); (lanes e and f) $A^+$RNA from normal splenocytes (4 and 5.5 μg, respectively); (lanes h and j) MOPC-41 $A^+$ microsomal RNA (0.3 and 0.6 μg, respectively). (B) Hybridization to a $C_\mu$ probe from pABμ-1 (the second time this blot has been hybridized). (D) Hybridization with a $C_\alpha$ probe from pα8 (the fourth time this blot was hybridized). (C) Autoradiograph of hybridization to a nitrocellulose Nothern blot with a probe from $P\gamma_{2b}(11)^7$. Lanes contained the following: (lanes a and e) normal thymocyte $A^+$RNA (5 and 10 μg, respectively); (lane b) immune thymocyte $A^+$RNA (5 μg); (lane c) normal splenocyte $A^+$RNA (5 μg, partially degraded); (lane d) immune splenocyte $A^+$RNA (5 μg); (lane f) no RNA; (lane g) $\gamma_{2b}$ mRNA (30 ng). Lines to the left of each figure represent the positions of 28S and 18S RNAs.

sibility; these products could represent translations of different $\gamma_1$-mRNAs analogous to the multiple μ-mRNAs found in 70Z/3 cells (Perry et al., 1979). Such mRNAs could have different 3′ ends, one for a membrane-bound protein and one for a secretory protein, or different signal sequences at the 5′ ends.

The μ band migrates significantly faster than the mature chain visible in sera of mice with the PC 3741 myeloma and in comparison to the mature μ 3741 of 75000 daltons reported by others (Marcu et al., 1978). The smaller size is likely due to lack of carbohydrate moieties compared with the mature polypeptides. Of interest is the loss of μ-mRNA in PC 3741 after only a few tumor passages with subsequent ability of $A^+$RNA to code for a new polypeptide band, μ′ (Figure 1A, lane F). Although we have no evidence that this band contains μ peptides, it is possible and would be analogous to the findings of other laboratories inferring that DNA rearrangements affect Ig production and the production of aberrant Ig chains in plasmacytomas (Kenter & Birshtein, 1979; Morrison, 1979).

The cDNA probes are mostly representative of the C region, with the remainder consisting of 3′-untranslated sequences (Table I), as are the cloned probes [pABμ-1 is 80% and $p\gamma_{2b}(11)^7$ is >90% C region]. Since the cDNA and cloned probes represent mainly the C region and since hybridization plateaus reached 75–100%, we are essentially examining thymocytes for the presence of C-region RNA sequences. The cDNAs have not been directly tested for class specificity by cross-hybridization with other heavy-chain mRNAs; however, we used the same myelomas and method of cDNA preparation for mRNA as Marcu et al. (1978). These authors showed that there was a high level of specificity. The cDNA probes do not hybridize to κ-mRNA, the other major mRNA present in the myelomas used to purify the heavy-chain mRNA templates, and contain less than 15% double strandedness (not shown). The ssDNA probes contain almost no double strandedness (Figure 4) and should be close to 100% pure probes. These probes have detected μ-RNA sequences, and some $\gamma_1$- or $\gamma_{2b}$-RNA sequences in normal thymocytes. There are several strong arguments why the presence of the heavy-chain RNAs does not result from peripheral plasma cell contamination [plasma cells are of more concern than B cells since they may contain 10000 or more copies of an Ig-mRNA while B cells have little more Ig-RNA than T cells (Storb et al., 1976, 1977)]: (1) Immunofluorescence with an anti-Ig antiserum indicates only about 0.02% plasma cell contamination by cytoplasmic staining. (2) Hyperimmunization increases $\gamma_1$-RNA in the spleen by about 100-fold, but $\gamma_1$-RNA in thymus barely rises, indicating minimal infiltration with $\gamma_1$-containing plasma cells. (3) The thymus has about 70 μ-RNA molecules per average cell, about 0.5–0.3 the amount per average splenocyte (it is very unlikely that the thymus contains 0.5–0.3 as many plasma cells as the spleen). (4) μ, α, and γ as well as κ polypeptide chains are present in thymus cells even after concanavalin A treatment with concomitant further reduction of the levels of B cells and plasma cells (Putnam et al., 1980). (5) Recent experiments have shown that μ genes are in an active, DNase I sensitive conformation in thymus T cells (U. Storb and R. Wilson, unpublished experiments). The active genes of the small number of contaminating B cells and plasma cells would not be detectable.

Most of the μ-RNA is present in the thymus cytoplasm (Table II). Further, the μ-RNA (and α-RNA) molecules are at least 50% polyadenylated (R. I. Near, unpublished experiments). Northern blots show that the μ-RNA in thymocytes is of the same size as that in splenocytes (as are α- and κ-RNAs). The diffuseness of the μ-RNA band is suggestive of μ-RNA species of more than one size as has been found by others both in thymocytes (Kemp et al., 1980) and in B cells (Rogers et al., 1980). The data support the possibility that thymocyte μ-RNA (as well as κ- and α-RNAs) may be functional mRNAs which may be translated by thymocytes (Putnam et al., 1980). The observation that μ-RNA of thymus is heterogeneous in size while the other Ig RNAs of the thymus are homogeneous in size, when analyzed with the same blot or the same thymocyte RNA, suggests functionalities of μ-RNA distinct from those of α- or κ-RNA.

One surprising result is the magnitude of the $\gamma_1$-RNA response in splenocytes relative to μ- and $\gamma_{2b}$-RNAs after immunization with SRBC (Table II). While $\gamma_1$-RNA increases about 100-fold, μ- and $\gamma_{2b}$-RNAs increase by 2–3-fold and 3–4-fold, respectively. Honjo et al. (1977) have done analogous studies with $\gamma_1$- and κ-RNAs in spleen after SRBC immunization. They find that IgM-producing cells in the spleen decline after 3 days postimmunization, and by 7 days they are only a little above background, while $\gamma_1$-producing cells and $\gamma_1$-RNA are increasing up to 7 days postimmunization. In general, the $\gamma_1$ response to immunization is more pronounced

than the $\gamma_2$ response (Plotz et al., 1968), and, depending on the mouse strain, antigen, method, and timing of immunization, the $\gamma_1$ response can be much greater than the $\gamma_2$ response (Barth et al., 1965; Pierce et al., 1972). In certain experimental conditions, germ-free BALB/c mice can have greater than 100-fold more IgG1 than IgG2 in the serum (Lawton et al., 1972). Therefore, we do not consider the $\gamma_1$ response necessarily excessive. The higher levels of $\mu$-RNA in the normal spleen and the low levels of $\gamma_1$- and $\gamma_{2b}$-RNAs correlate with the high percentage of surface IgM positive spleen cells (about 38%) and the low percentage of IgG2 (2.1%) and IgG1 (0.5%) positive cells (Abney et al., 1978). The high level of $\mu$-RNA in the thymus, of course, does not reflect Ig-positive cells. The levels of Ig-RNAs in spleen compared to thymus are roughly similar whether estimated by densitometer tracings of Northern blots or quantitated by hybridization kinetics. The ratios of spleen/thymus Ig-RNAs are 4, 3.5, not determined, and 1 as determined by Northern blots, and by hybridization kinetics they are 2, 2.5, not calculable, and 2 for $\kappa$, $\mu$, $\gamma_{2b}$, and $\alpha$, respectively. The quantitation by hybridization kinetics is probably more accurate in both an absolute and a relative sense since the kinetics are not subject to electrophoresis artifacts, nonlinearity of film, variability of transfer of RNA onto blots, and possible losses of RNA or unknown content of rRNA after oligo(dT)–cellulose chromatography. The presence of $\gamma_{2b}$-RNA in normal thymocytes as detected by Northern blots disagrees with the hybridization kinetics to a small extent. At about 5–10 molecules/cell, the kinetics often do not reach a plateau whereas it is still possible to detect $\gamma_{2b}$-RNA from thymocytes on Northern blots as seen in Figure 6C (lane a). Such a small amount of $\gamma_{2b}$-RNA in thymocytes may be due to contamination with plasma cells, low levels of $\gamma_{2b}$-RNA in thymocytes, or subpopulations of T cells with significant quantities of $\gamma_{2b}$-RNA. These possibilities have yet to be distinguished. Immunization, in all cases, leads to the intensification of bands in both splenocyte and thymocyte RNAs. The Northern blots show $\gamma_{2b}$-RNA in thymocytes to increase by 4-fold and $\kappa$-RNA to increase by 2-fold. The immunofluorescence indicates, however, that this immune thymocyte preparation has one-third fewer plasma cells than the normal thymocytes used for these blots. Thus, an increase of Ig-RNA in thymocytes probably reflects an intrinsic increase in the thymocytes themselves. This is analogous with studies of concanavalin A stimulated thymocytes metabolically labeled with radioactive amino acids which show increases in the production of both the $\kappa$ chain and a $\gamma$-like chain (Putnam et al., 1980) while contaminating B cells or plasma cells decreased.

Recently, another group of investigators (Kemp et al., 1980) reported the presence of $\mu$-RNA in CBA mouse thymocytes as assayed by Northern blots. Further, they reported only small amounts of $\kappa$-RNA in their crude thymocyte preparation and essentially no $\kappa$-RNA in thymocytes treated with anti-Ia antiserum plus complement. In view of the fact that they loaded 10 times more thymus RNA than spleen RNA onto gels, the difference between spleen and even "crude" thymus is impressive. Our results are quite different in that by kinetics splenocytes have only about twice as much $\kappa$-RNA as thymocytes (Near & Storb, 1979) and by blots about 4 times as much $\kappa$-RNA. The discrepancy between these two sets of data may be due to the strain of mice used. We have recently found that CBA thymuses contain many times less $\kappa$-RNA than BALB/c thymuses (K. Ritchie and U. Storb, unpublished experiments).

We have extensively reviewed the protein–biochemical data supporting T-cell Ig in previous articles (Storb et al., 1976, 1980; Putnam et al., 1980; Near & Storb, 1979). Briefly, there is some evidence for $\mu$ and $\alpha$ chains associated with thymocytes which may be immunoprecipitated and display the correct electrophoretic mobility (Moroz & Lahat, 1974; Cone & Marchalonis, 1974; Putnam et al., 1980; Hämmerling et al., 1976a,b). These results have been obtained both with normal thymocytes and with cloned lymphoma lines containing $\theta$ antigen (Marchalonis et al., 1972; Harris et al., 1973). These results are not reported in all laboratories, many of which postulate C regions unique to T cells, IgT (Cone, 1977).

Many laboratories, nevertheless, agree that T cells bear idiotypic determinants similar to, if not identical with, those present in serum Ig (Binz et al., 1979; Weinberger et al., 1979; Binz & Wigzell, 1975; Black et al., 1976). The putative T-cell antigen receptor has been isolated in several laboratories (Krawinkel et al., 1977; Binz & Wigzell, 1976b) as have several idiotypically positive T-cell factors (Germain et al., 1979; Bach et al., 1979). These studies present evidence for receptors or factors containing idiotypic determinants linked to heavy-chain allotypic markers, but not usually possessing Ig C-region determinants (although some helper factors may be exceptions; Taniguchi & Tada, 1974). The T-cell receptor isolated by Binz & Wigzell (1976b) is the correct size to be an Ig heavy chain (70 000 daltons).

Most of these studies involve T-cell populations which are mature and functional in such activities as killing or suppression. The present study, however, concerns thymocytes (thymus cells), the great majority of which (>90%) are immature, immunoincompetent, and high-density Thy-1 T cells (Leckband & Boyse, 1971). These cells are in an earlier stage of development than the functional T cells described above. It is quite possible that T cells undergo a developmental program involving the initial synthesis of Ig polypeptides possessing classical C regions followed, upon maturation, by a switching to a T-cell specific C region. Such switching of C regions at the DNA level has already been noted for Ig heavy-chain classes in plasma cells (Kataoka et al., 1980; Coleclough et al., 1980; Cory & Adams, 1980).

However, other possibilities may also account for the difficulty in detection of T-cell Ig. Posttranslational modifications such as glycosylation may alter the antigenicity of T-cell Ig such that conventional antisera may not detect it. Further, detergent concentrations are apparently critical and distinctly different for isolation of B-cell Ig and T-cell Ig (Cone & Brown, 1976).

The high level of $\mu$-RNA, its possession of poly(A), its location within the cytoplasm, and its correct size compared to splenocyte $\mu$-RNA imply its use as a functional mRNA in thymocytes. $\gamma_1$- and $\gamma_{2b}$-RNAs do not exist in the majority of thymocytes in large amounts but may be present in a small number of thymocytes. The results concerning $\gamma_{2b}$-RNA may apply to $\gamma_{2a}$-RNA since these subclasses display about 60% cross-hybridization (Yamawaki-Kataoka et al., 1979). The putative function of the immunoglobulin RNAs would be to code for an Ig polypeptide for use as an antigen receptor. This receptor need not be permanent, but may change Ig class to a T-cell specific class at a later stage of maturation.

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# Fourth Component of Human Complement: Studies of an Amine-Sensitive Site Comprised of a Thiol Component†

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ABSTRACT: Studies of human C3 following treatment with nitrogen nucleophiles revealed the presence of an amine-sensitive bond whose properties were consistent with an internal thiol ester [Janatova, J., Lorenz, P. E., Schechter, A. N., Prahl, J. W., & Tack, B. F. (1980a) *Biochemistry 19*, 4471–4478; Janatova, J., Tack, B. F., & Prahl, J. W. (1980b) *Biochemistry 19*, 4479–4485; Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., & Prahl, J. W. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5764–5768]. The selected studies revealing the generation of a reactive carbonyl and a sulfhydryl group in C3 were extended to C4 and C5. As with C3, the reaction of methylamine with native C4 is stoichiometric, covalent, accompanied by the appearance of a sulfhydryl group, and results in the loss of C4 hemolytic activity. Electrophoretic and autoradiofluorographic analyses of radiolabeled ([$^{14}$C]-methylamine or [$^{14}$C]iodoacetamide) C4 samples have shown that the reactive carbonyl and resultant sulfhydryl group both reside in the $\alpha$-polypeptide chain. A sulfhydryl group was also detected following cleavage of native C4 by $C1\bar{s}$, a serine protease responsible for activation of the C2 and C4 components, and was shown to be present in the $\alpha'$ chain of C4b. Inactivation of C4 by treatment with nitrogen nucleophiles or as it naturally occurs on storage is apparently accompanied by physicochemical changes in the molecule. Hemolytically inactive forms of C4 can be separated from native C4 by chromatography on diethyl(2-hydroxypropyl)aminoethyl-Sephadex A-50, and all chromatographic forms exhibit the same $\alpha\beta\gamma$ polypeptide chain structure. In contrast to native C4, hemolytically inactive forms are not cleaved by $C1\bar{s}$ and fail to undergo a denaturant-induced $\alpha$-chain fragmentation. The autolytic cleavage of the C4 $\alpha$ chain, accompanied by the expression of a sulfhydryl group, is a property of native C4 seen when the protein is incubated in sodium dodecyl sulfate or guanidine hydrochloride. The cleavage reaction results in two fragments, $C4_\alpha$-40 000 and $C4_\alpha$-54 000, with the sulfhydryl group present in the N-terminal, lower molecular weight fragment. This reaction can be prevented either by the presence of $\beta$-mercaptoethanol or by prior nucleophile or spontaneous inactivation. In contrast to C3 and C4, the hemolytic function of C5 is not affected by treatment with amines, and C5 does not undergo autolytic cleavage. Data presented here are interpreted to indicate the presence of an internal thiol ester in C4 and the absence of such a bond in C5. The relationship between this site and the covalent association of C4b with red cell membranes and immune aggregates is discussed within the context of a transesterification reaction.

The human complement proteins C4, C3, and C5 are present in plasma in precursor forms as disulfide-bridged subunit structures. A three-polypeptide chain structure, $\alpha\beta\gamma$, exists in C4 (Schreiber & Müller-Eberhard, 1974), while C3 and C5 are comprised of two polypeptide chains each, $\alpha$ and $\beta$ (Nillsson & Mapes, 1973). Chemical and biological properties of these proteins have been reviewed in detail (Müller-Eberhard, 1975; Porter & Reid, 1979; Stroud et al., 1979). The activation of the native C4, C3, and C5 is a sequential enzymatic process in which the proteins are first converted at the membrane surface by a limited proteolysis into a transient "activated" state. This activation step results in production of two fragments. The smaller one (C4a, C3a, and C5a) is released from the N terminus of the respective $\alpha$ chain and is referred to as an activation peptide with anaphylatoxic activity (Gorski et al., 1979; Bokisch et al., 1969; Hugli et al., 1975; Fernandez & Hugli, 1978). The larger fragment (C4b or C3b) contains the labile binding site, and consequently can form a covalent association with receptor(s) on a plasma membrane, cell wall, or immune aggregate. The surface-bound fragment with "active" configuration functions to recruit and participate in the activation of the next complement component in the reaction sequence. C4b and C3b are subcomponents of the classical pathway C3- and C5-converting enzymes ($\overline{C4b2a}$ and $\overline{C4b2a3b}$, respectively) which are assembled on the particle surface (Müller-Eberhard et al., 1967). C3b further functions as a subcomponent of the C3 and C5 con-

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