

Sequences related to immunoglobulin kappa chain messenger RNA in T cells

[B ("bursa" dependent) and T (thymus derived) cells/RNA-DNA hybridization/complementary DNA]

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ABSTRACT We investigated by molecular hybridization whether T cells contain RNA sequences homologous to RNA which codes for immunoglobulin kappa-chain (κ -chain). A radioactive probe of complementary DNA (cDNA) was prepared by transcription of purified κ -chain mRNA from mouse myeloma MOPC-41 with reverse transcriptase (RNA-dependent-DNA nucleotidyltransferase) from avian myeloblastosis virus. The cDNA probably corresponded only to the constant region and 3'-terminus of κ -chain mRNA. Kappa-chain cDNA was found to hybridize efficiently with RNA from both thymus cells and an established culture of thymoma cells. The thymus and thymoma cells contained 99.8% and 100% theta-positive cells, respectively. Quantitatively the average thymus T cell (thymus derived lymphocyte) contained about one half as much κ -chain mRNA as the average spleen B cell ("bursa" dependent lymphocyte), whereas the thymoma cells contained only 1/33 as much. Control hybridizations of κ -chain cDNA with myeloma and liver RNA support the conclusion that T cells in the thymus and in the thymoma cell line synthesize κ -chain mRNA-like molecules. The thermal stability of hybrids of κ -chain cDNA with RNA from spleen, thymus, thymoma, and another κ -chain producing myeloma tumor was lower than that with MOPC-41 RNA. This finding may be due to the existence of several slightly different *c_κ* genes in the mouse as suggested by various control experiments.

Thymus derived lymphocytes (T cells) are responsible for cell mediated immune responses and "helper" functions in the induction and/or regulation of humoral antibody synthesis (1). These functions appear to be antigen specific and therefore require an antigen recognition system (2, 3). Although the antigen receptor on B cells ("bursa" dependent lymphocyte) is well defined as membrane bound Ig molecules, T cell receptors have not been conclusively proven or identified (4).

T cells have been analyzed by a variety of methods in the search for Igs with controversial results. Indirect immunofluorescence assays of T cell membranes with anti-Ig antisera have yielded both positive (5, 6) and negative (7, 8) results. By radioimmune assays, extremely small amounts of T cell surface Ig have been reported (9, 10).

It has been suggested that the difficulty in binding anti-Ig antibodies to the T cell may be due to the fact that the Ig molecule was buried in the membrane with the Fc portion unexposed so that it is effectively "masked" (11). In order to circumvent this problem, T cell membranes were solubilized after lactoperoxidase-catalyzed iodination of the surface proteins, and the presence of Igs determined by immunoprecipitation. Marchalonis *et al.* reported successful isolation of T cell Igs by

Abbreviations: T cell, thymus derived lymphocyte; B cell, "bursa" dependent lymphocyte; cDNA, complementary DNA; c-region, constant region of antibodies; C_t , concentration of RNA in hybridization reaction \times the time of incubation, expressed as mol of nucleotide \times sec/liter; $C_{t1/2}$, C_t at which the hybridization reaction has proceeded to one half completion; PB, phosphate buffer of equimolar mixture of Na_2HPO_4 and NaH_2PO_4 at pH 6.8; t_m , temperature at which 50% of the hybrids have melted.

this method (12, 13). Other investigators were, however, unable to reproduce this finding (14, 15).

The chief criticism of positive findings has been that the amounts of Igs detected are so small that the significance is questionable. Furthermore, in all of these studies the possibility existed that the Igs had been absorbed passively by the T cells. To meet this criticism the incorporation of labeled amino acids into Igs was studied. Moroz *et al.* reported that 15% or more of T cell protein synthesis was devoted to Igs (16, 17), whereas others found no evidence for Ig synthesis (15, 18).

In the present study we circumvented the problems which accompany the studies of Ig proteins on T cells by searching for the presence of RNA sequences in thymus cells homologous to Ig mRNA. We have limited our study to kappa-chain (κ -chain) mRNA, since 90-95% of mouse L-chains are of the kappa type. It appeared most likely that if T cells produce conventional Igs they would also contain κ -chains. We have used very sensitive molecular hybridization techniques with DNA complementary (cDNA) to the constant region of mouse κ -chain mRNA. With this tool the presence and quantities of RNA sequences homologous to κ -chain mRNA were investigated in thymus cells and spleen cells in which we knew the proportions of B and T cells, in a cultured thymoma cell line, and in control tissues.

MATERIALS AND METHODS

Preparation of cDNA. Purified globin mRNA was prepared from reticulocytes of Swiss mice as described (Storb and Marvin, unpublished). Purified κ -chain mRNA was prepared from microsomal fractions of MOPC-41 myeloma tumors from Balb/c mice as described (36). The κ -chain mRNA preparation used as a template for cDNA in this study was obtained by isolating, from sucrose gradients, a 13S peak of poly(A) RNA from MOPC-41 microsomes (36), and further purifying it by preparative polyacrylamide gel electrophoresis on 2.2% disk gels. The final RNA preparation migrated as a single 13S band by analytical electrophoresis on polyacrylamide gels. The RNA strongly stimulated the cell free synthesis of κ -chain precursor in a wheat germ cell-free system (19, 36).

cDNA was prepared from κ -chain and globin mRNA by the use of reverse transcriptase RNA-dependent-DNA nucleotidyl transferase of avian myeloblastosis virus as described by Verma *et al.* (20). The label was [^3H]dCTD with a specific activity of 25 Ci/mmol (New England Nuclear). The globin and κ -chain cDNAs sedimented as a single peak in alkaline sucrose gradients with an $s_{20,w}$ of approximately 5.5.

Preparation of Whole Cell RNAs. Total RNA was extracted from cells or tissues as described (21). To permit the enumeration of B and T cells, single cell suspensions were prepared from spleens and thymuses as follows. The tissues were coarsely minced with scissors and washed several times in ice-cold physiologic saline to remove contaminating blood. After further

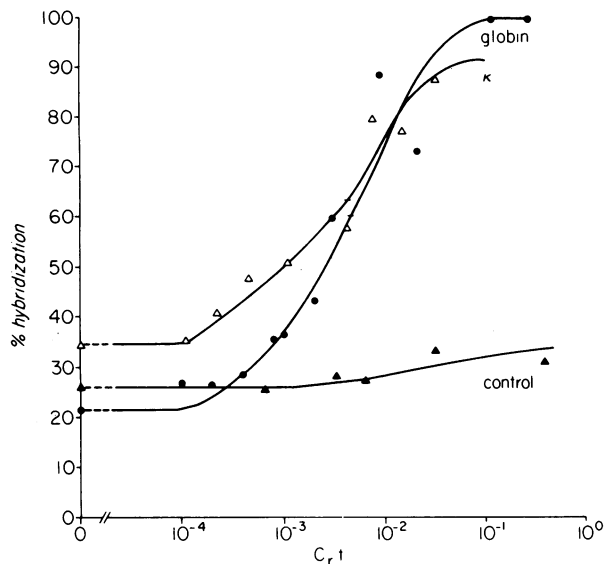


FIG. 1. Hybridization of κ -chain and globin cDNA with their respective mRNAs. κ -chain [^3H]cDNA (Δ) or globin [^3H]cDNA (\bullet) was hybridized with an excess of unlabeled mRNA as described in *Materials and Methods*. (Δ) control: hybridization of κ -chain [^3H]cDNA with an excess of globin mRNA.

rinsing in saline, the tissue pieces were gently ground in a loose fitting glass homogenizer to release the mobile cells. Pieces of connective tissue were then removed by sieving the cells through nylon gauze. An aliquot of the resulting cell suspension was tested for content of B and T cells by immunofluorescence (see below) while the bulk of the cells were used for RNA extraction.

A tissue culture line of thymoma cells was obtained as follows. A thymoma which arose spontaneously in a male C3H mouse was serially passaged in C3H mice by subcutaneous inoculations. After the third subcutaneous passage, tumor cells were inoculated intraperitoneally, and cultures were set up from the resulting ascites in Dulbecco's modified Eagle's medium with 10% heat-inactivated horse serum. The thymoma cells used in the present study had been in continuous culture for more than 3 months. The generation time was approximately 24 hr. The cells grew to a maximal density of 7×10^6 cells per ml.

The final RNA preparations were tested for residual DNA content by the diphenylamine reaction. The thymoma, thymus, and spleen RNA preparations used in this study contained 0%, 1.6%, and 0.7% DNA, respectively.

Enumeration of T and B Lymphocytes. The presence of theta and Ig surface markers on spleen, thymus, and thymoma cells was analyzed by immunofluorescence (Table 1). Fluoresceinated immunoglobulins isolated from a rabbit antiserum directed against mouse brain were used to detect murine T lymphocytes (22). A goat immunoglobulin preparation containing fluorescein-labeled anti-mouse Ig was used to identify immunoglobulin bearing B lymphocytes.

cDNA-RNA Excess Hybridization. cDNA (500–800 cpm/aliquot) was mixed with its mRNA template in 10- to 200-fold molar excess, or with 1 μg (MOPC-41) to 200 μg (liver) of whole cell RNA per aliquot in 0.24 M equimolar phosphate buffer at pH 6.8 (PB) with 0.5 mM EDTA. The mixtures were divided into 20 μl aliquots, sealed in capillaries, boiled for 5 min, and incubated at 67°. At the desired times, aliquots were quickly frozen. All aliquots from one experiment were analyzed together by digestion with S_1 nuclease (Miles Laboratories) (23).

Thermal Denaturation of Hybrids. Hybridization mixtures

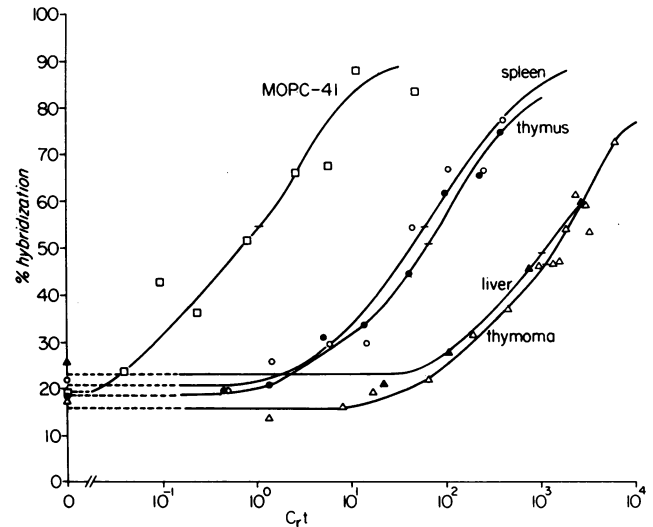


FIG. 2. Hybridization of κ -chain cDNA with whole cell RNAs. κ -chain [^3H]cDNA was hybridized with an excess of RNA as described in *Materials and Methods*. Whole cell RNAs: \square , MOPC-41; \circ , Swiss spleen; \bullet , Swiss thymus; Δ , C3H thymoma tissue culture; \blacktriangle , Balb/c liver. Background S_1 nuclease resistance is shown at $C_r t$ 0. The $C_r t_{1/2}$ values are indicated by horizontal lines. For the calculation of the $C_r t_{1/2}$ of liver RNA, it was assumed that 90% of the hybridization would be completed within 100 $C_r t$.

sealed in capillaries were incubated at 67° long enough to achieve maximal hybridization. Four capillaries were then quickly frozen to be used for the determination of total trichloroacetic acid (TCA) precipitable radioactivity and the percentage of nuclease resistant radioactivity. Melting profiles were obtained by raising the temperature in 5° increments and quickly freezing duplicate capillaries at each step for the determination of S_1 nuclease resistant radioactivity.

RESULTS

The κ -chain cDNA probe

The purity of the κ -chain cDNA probe was assessed by comparing its hybridization kinetics with κ -chain mRNA to that of globin cDNA with globin mRNA (Fig. 1). The κ -chain cDNA and globin cDNA hybridized with their respective mRNAs with a $C_r t_{1/2}$ of 4.3×10^{-3} and 4.6×10^{-3} , respectively. This indicates that the κ -chain cDNA probe is essentially pure (see *Discussion*). As a specific control, κ -chain cDNA was incubated together with globin mRNA; no significant hybridization was observed.

The variable background observed for radioactivity that was S_1 nuclease resistant in the hybridization mixtures is apparently dependent on the RNA used. In the absence of RNA less than 10% of the radioactivity in cDNA was S_1 nuclease resistant.

Hybridization of κ -chain cDNA with whole cell RNAs

The relative quantities of κ -chain RNA in different cell populations were determined by hybridizations of κ -chain cDNA with whole cell RNAs. Fig. 2 shows that both the thymus and the thymoma culture cells which contained very few or no B cells (Table 1) possess significant amounts of κ -chain RNA. The total spleen contains 1.1 times as much κ -chain RNA per total RNA as the thymus. On the other hand, a myeloma tumor, in which approximately 20% of the total protein synthesis is devoted to κ -chains (24), contains 59 times as much κ -chain RNA per total RNA as the thymus cells (Fig. 2). As another control, liver RNA was hybridized because this organ is very vascular

Table 1. Immunofluorescence of B and T cells used for RNA extraction

Cells	% Theta-positive	% Ig-positive
Spleen	31	42
Thymus	99.8	0.2
Thymoma	100	0

and therefore rich in circulating lymphocytes. It contained only about one sixteenth the amount of κ -chain RNA per total RNA compared with the thymus. The hybridization with liver RNA was not carried to completion, and the $C_{rt_{1/2}}$ value is therefore only approximate. The $C_{rt_{1/2}}$ of hybridization with the various RNAs and the relative amounts of κ -chain RNA are summarized in Table 2.

The relative quantities of κ -chain RNA in B and T cells were calculated on the basis of the data in Tables 1 and 2: the spleen contains 31% T cells, 42% B cells, and 1.1 times as much κ -chain RNA as the thymus; the thymus contains 99.8% T cells and 0.2% B cells. Thus, 31 T + 42 B = 1.1, and 99.8 T + 0.2 B = 1. Therefore, B/T = 1.88, i.e., the average B cell contains 1.88 times as much κ -chain RNA as the average T cell.

Thermal denaturation of hybrids

Fig. 3 shows the melting profiles of κ -chain cDNA hybrids with various RNAs and DNAs. The homologous reaction with κ -chain RNA and total MOPC-41 RNA shows a very sharp thermal denaturation profile with a t_m of 93° and 92°, respectively. The hybrids with whole cell RNA from MOPC-41, in addition, show approximately 15% of a lower melting component. Such a lower melting component was also present in the hybrids between κ -chain cDNA and whole cell RNA of another κ -producing myeloma, 66.2 (data not shown).

The major transition with the latter RNA was at 87.7°. The hybrids with Swiss spleen, Swiss thymus, and the thymoma RNA melt 8.5 to 11.7° below the t_m of hybrids with homologous MOPC-41 RNA. This is apparently not due to strain differences, since the hybrids with Balb/c spleen RNA denature also with a low t_m of 82°. Hybrids of κ -chain cDNA and homologous Balb/c or heterologous Swiss DNA show indistinguishable melting profiles with a t_m of 90°.

DISCUSSION

The major prerequisite for the validity of the finding of κ -chain mRNA-like sequences in T cells is the availability of a pure probe of κ -chain cDNA. Before using the cDNA reported here, we tested a variety of other κ -chain cDNAs. The $C_{rt_{1/2}}$ of κ -chain cDNA/ κ -chain mRNA hybrids was compared with that of globin cDNA/globin mRNA hybrids. It was found that the κ -chain cDNA hybridized with its template mRNA three to ten times more slowly than globin cDNA/globin mRNA and did not show pseudo first-order kinetics unless the κ -chain mRNA was highly purified by several sucrose gradient centrifugations and/or polyacrylamide gel electrophoresis.

The κ -chain cDNA used in the present study hybridized with κ -chain mRNA with satisfactory kinetics (Fig. 1). The rationale for using globin cDNA as a kinetic standard has been discussed by Leder *et al.* (25). In our experiments, the κ -chain cDNA and globin-cDNA were approximately of the same size. mRNA for α - and β -globin together correspond to approximately 1200 nucleotides in length (25); so does the κ -chain mRNA (36). It should, therefore, be expected that if the κ -chain cDNA is pure it will hybridize with its template RNA with the same $C_{rt_{1/2}}$ as globin-cDNA does with its template. This was shown to be

Table 2. Relative amounts of κ -chain RNA in different cells

RNA	$C_{rt_{1/2}}$	% κ -chain RNA*
κ -chain mRNA	4.3×10^{-3}	100
MOPC-41	1.05	0.4100
Spleen	59	0.0073
Thymus	65	0.0066
Thymoma	1100	0.0004
Liver	1020	0.0004

* The % κ -chain RNA in the various tissues was calculated as the ratio of the $C_{rt_{1/2}}$ of a given RNA relative to the $C_{rt_{1/2}}$ of purified κ -chain mRNA.

the case (Fig. 1). It was assumed that the globin cDNA was of high purity, since globin mRNA is the major poly(A)-containing RNA species produced in reticulocytes, and it appears as a very sharp peak in sucrose gradients of reticulocyte poly(A)-containing RNA, which can be easily separated from other RNA species. The globin mRNA used as a template for cDNA stimulated the synthesis of globin in a cell free wheat germ system, and of no other completed proteins (36).

We presume the κ -chain cDNA to correspond only to the 3'-portion of the κ -chain mRNA for the following reasons. The primer used in the synthesis of cDNA was oligo(dT). It is therefore most likely that the transcription was started from the 3'-poly(A) terminus of the κ -chain mRNA molecule (25-27). The resulting cDNA sedimented in alkaline sucrose gradients at 5.5 S which corresponds to a molecular weight of about 110,800 or approximately 369 nucleotides (28). This length is enough to cover the untranslated 3'-end and a portion corresponding to the constant region of the κ -chain mRNA (29). The κ -chain cDNA is, however, probably not long enough to contain

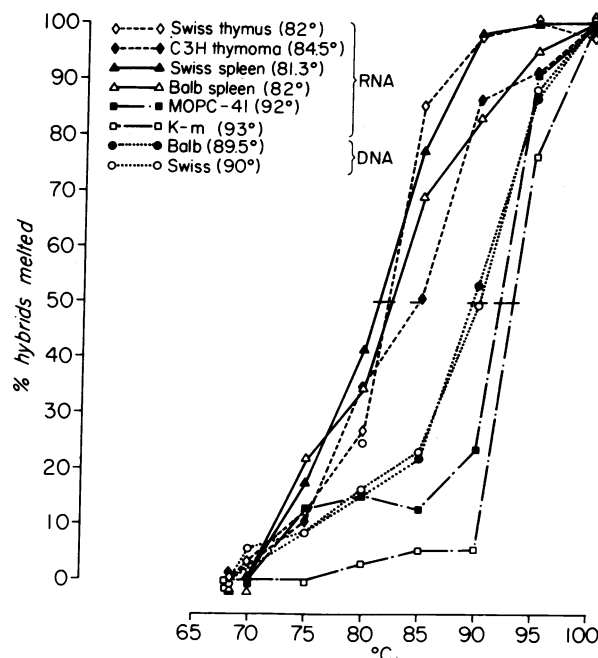


FIG. 3. Thermal stability of hybrids between κ -chain cDNA and various RNAs or DNAs. The methods are described in the text. The RNAs were hybridized to completion (see Figs. 1 and 2). The Balb/c DNA (MOPC-41) and Swiss DNA (Krebs ascites cells) were sheared in the French press to a single strand size sedimenting at 10.1 S in alkaline sucrose gradients; DNA/cDNA ratios were approximately 4×10^6 ; at C_{ot} 5000, 56.8 and 59.5% of the cDNA was hybridized with the DNAs. t_m s are shown in parentheses.

variable region homologues. It is, therefore, an ideal probe to search for κ -chain mRNA in various tissues because, regardless of the variable regions expressed, all κ -chain mRNAs should hybridize with the cDNA.

The data shown in Fig. 2 indicate clearly that cells in the thymus and in a thymoma contain RNA sequences related to κ -chain mRNA. We will use the term κ -chain RNA in the remainder of the discussion since whole cell RNAs were used and the function of the hybridizing sequences is yet unknown.

The hybridization found between κ -chain cDNA and thymus or thymoma RNA could not have been due to contamination of the RNA probes by cellular DNA. The thymoma RNA was free of DNA (0.5% would have been detected). The thymus RNA contained 1.6% DNA; if one assumes a maximum of 10 c_κ genes per haploid genome (25–27), this amount of DNA would have been far too little to hybridize with any significant amount of the cDNA, since the cDNA/DNA sequence ratio would have been 2000.

The evidence that κ -chain RNA is present both in T cells and B cells is as follows. The thymus cell preparation had been shown to contain 99.8% theta-positive cells and only 0.2% Ig-positive cells (Table 1). The Ig-positive cells were probably derived from blood contamination, and 0.2% is a very small amount indeed. On the other hand, "liver" RNA contained only one sixteenth the amount of κ -chain RNA compared with the thymus. Liver RNA was included as a control to assess the maximal amount of κ -chain RNA which could be contributed by circulating blood lymphocytes. The liver is much more vascular than the thymus and no attempt had been made to remove blood from the liver preparation. It is therefore probable that the few B cells contaminating the thymus preparation would have contributed only a very small proportion of the total κ -chain RNA found in this organ. Furthermore, the MOPC-41 myeloma which is highly active in the synthesis of κ -chains, contains only 59 times as much κ -chain RNA as the thymus. Thus, the 0.2% B cells contaminating the thymus (i.e., 1/500 of the total cell population) would not have contained sufficient κ -chain RNA even if they had been cells of as high an activity as myeloma cells. Lastly, the thymoma culture cells were a pure population of theta-positive cells, which nevertheless contained significant amounts of κ -chain RNA. The latter is only suggestive evidence, since the tumor cells may express genes which are otherwise silent.

If one assumes, however, that the thymoma cells produce κ -chain RNA in quantities equivalent to the normal T cell from which the malignant line was derived, the question remains, why the thymoma cells produce 17 times less κ -chain RNA than the thymus. One possible explanation is that the tumor cells are a very immature T cell population, maybe even less mature than the average thymus cortex cell. We have preliminary evidence that this is the case. The thymoma cells appear to contain more theta antigen than the average thymus cell as judged by the strength of the immunofluorescence with anti-brain serum (Clagett, Buck, and Storb, unpublished). The thymoma cells are also highly sensitive to killing by small quantities of phytohemagglutinin (30; Buck and Storb, unpublished). It appears possible therefore that T cells accumulate increasing quantities of κ -chain RNA as they mature.

The average B cell was found to contain only 1.88 times as much κ -chain RNA as the average T cell. It must be emphasized that this is an average value and that the range of quantities of κ -chain RNA within the B and T cell populations cannot be estimated at present. Nevertheless the result is unexpected since the quantities of Ig, if any, observed associated with T cell membranes was over 100 times less than membrane bound Ig

in B cells (31). The discrepancy may be due to lack of transport of κ -chain RNA into the cytoplasm, inefficient translation, rapid catabolism of κ -chains, or a different mechanism of incorporation of Ig into the membrane of T cells.

All the RNAs tested, except the homologous whole cell tumor RNA (MOPC-41), formed hybrids with κ -chain cDNA which were less well matched than the hybrids with the homologous κ -chain mRNA (Fig. 3). The t_m s were 5.3–11.7° lower. This corresponds to a maximum of 5.3–11.7% mismatching (32), and probably less because our experiments were carried out at higher salt concentrations (32). The following factors have to be considered as contributing to the decreased thermal stability of hybrids with the heterologous RNAs: impurity of the cDNA, size of the hybridizing RNAs, strain differences in the 3'-terminus of κ -chain RNA, and the presence of several slightly different c_κ genes.

It is unlikely that the hybrids with heterologous RNAs are largely due to reactions with non- κ sequences in the cDNA. As discussed above, the hybridization kinetics of κ -chain cDNA with κ -chain mRNA indicate a relatively high purity when compared with a globin-cDNA standard. Furthermore, the heterologous RNAs are able to anneal with essentially the total hybridizable cDNA.

The whole cell RNAs of thymuses and spleens were partially degraded to average sizes of approximately 14 S and 7 S (data not shown). The whole cell MOPC-41 RNA was essentially undegraded. To test whether size differences in this range of sizes would influence the thermal stability of hybrids, MOPC-41 whole cell RNA was mildly denatured by alkali to produce molecules of approximately 14 S and 7 S. Hybrids of such degraded MOPC-41 RNA with κ -chain cDNA melted with exactly the same kinetics and t_m as hybrids with undegraded MOPC-41 RNA (data not shown). That there is no influence by RNA size in these experiments may be due to the fact that all RNAs were larger than the cDNA probe.

The lowered thermal stability is apparently not due to strain-specific genetic differences in the κ genes of the mouse strains from which the κ -chain mRNA, or spleen, thymus, and thymoma RNAs, respectively, originated. First, the t_m of κ -chain cDNA hybrids with Balb/c spleen RNA is approximately the same as with Swiss spleen and thymus RNA. Second, there is no difference in the thermal denaturation of κ -chain cDNA hybrids with Balb/c or Swiss DNA.

It appears most likely that the heterologous RNAs differ from MOPC-41 κ -chain mRNA in the base sequence of the 3'-portions of the κ -chain RNAs. The greatest difference in t_m observed was 11.3° with spleen RNA versus MOPC-41 κ -chain mRNA. MOPC-41 κ -chain mRNA and spleen RNA differ, therefore, maximally by 11.3% of the base sequences. Thus, they are more closely related than κ - and λ -chain mRNAs, which differ by approximately 45% (33). The difference may either be located in the c-region, or the untranslated 3'-end of the κ -chain RNA (29), and probably in both since the spleen and thymus or thymoma RNA-cDNA hybrids lack the high melting component completely (Fig. 3).

The lower melting component present in whole cell MOPC-41 RNA- κ -chain cDNA hybrids may be due to infiltration of the tumor with host B and T lymphocytes. Their κ -chain RNA would not be present in purified myeloma κ -chain mRNA since the latter is produced from membrane bound ribosomes generally not present in untransformed B and T cells. The major thermal transition with MOPC-41 whole cell RNA has the same t_m as that with κ -chain mRNA, which suggests that the presence of other cellular RNAs does not interfere with faithful hybrid formation.

Also, the hybrids between MOPC-41 κ -chain cDNA and MOPC-41 DNA are mismatched. DNA-DNA hybrids generally melt with a t_m approximately 5° higher than RNA-DNA hybrids (34). However, the κ -chain cDNA-MOPC-41 DNA hybrids melt 3° below the t_m of κ -chain cDNA-MOPC-41- κ -chain mRNA hybrids. They are therefore mismatched by about 8%.

These results can be explained by postulating that there exist more than one, and possibly as many as 10 c_κ genes in the mouse which maximally differ by 11.3% in base sequence. There is evidence for a certain variability in c-regions from studies of human λ -chains where at least eight different types of constant regions have been found to date (35). Others (25–27) and ourselves (Wilson and Storb, unpublished) have found that κ -chain cDNA hybridizes with mouse DNA with unique kinetics. However, present methods of hybridization do not allow one to distinguish between one and up to 10 genes, since ideal kinetic standards are not available. Further experiments are required to clarify this point, which has interesting implications for the organization of immunoglobulin genes, and to rule out the possibility of other technical problems which have not been considered in the control experiments.

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