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Immunology

Transcripts of the immunoglobulin C_μ gene vary in structure and splicing during lymphoid development

(B, pre-B, T, and myeloid cells/gene activation/alternative 5′ and 3′ splices/intervening sequences)

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ABSTRACT **Two classes of transcripts of the mouse immunoglobulin μ constant region (C_μ) gene can be distinguished. Whereas B cells contain μ mRNAs of 2.4 and 2.7 kilobases (kb), many T lymphoma, Abelson pre-B lymphoma, and myeloid tumor cell lines contain polyadenylylated RNA species bearing C_μ sequences (C_μ RNAs) of 1.9, 2.1, 2.3, and 3.0 kb. Production of C_μ RNAs, unlike μ mRNAs, does not require recombination within the joining region (J_H) locus. To define the structure of C_μ RNAs, RNA from representative cell lines was fractionated by gel electrophoresis, transferred to diazobenzyloxymethyl paper, and tested for hybridization with 23 DNA fragments that collectively span a chromosomal μ gene, cloned from a plasmacytoma. All the C_μ RNAs bear sequences derived from each of the four C_μ domains, but none contain the intervening sequences separating domains; thus each represents a spliced RNA species. The 1.9-kb C_μ RNA contains the 3′ sequence characteristic of secreted μ chain, whereas the longer species bear that of membrane-bound μ chain. Hence C_μ RNAs and μ mRNAs are equivalent throughout the C_μ and 3′ terminal regions. They differ markedly, however, in their 5′ regions, because the 3.0- and 2.3-kb C_μ RNAs bear sequences from within the conventional J_H-C_μ intervening sequence. Because these sequences are several kb from C_μ, this region must contain at least one hitherto unsuspected splice site. C_μ RNAs may not express immunological diversity, because no evidence was found that they bear variable regions. T and pre-B lymphoid and myeloid cells contain equivalent C_μ RNA species, which coexist with μ mRNAs in some pre-B and B lymphoid lines. C_μ RNA expression appears to reflect an activated state of the C_μ gene common to cells at early stages of T, B, and myeloid development.**

Production of immunoglobulin heavy (H) chains by cells of the B lymphoid lineage requires recombination of gene segments and subsequent splicing of the primary transcripts to form mRNAs (reviewed in ref. 1). Early in the development of a B lymphocyte a functional μ heavy chain gene is formed by deletions (2, 3) that join one of many variable region (V_H) genes (4) to one of four joining region (J_H) genes near the μ constant region (C_μ) gene (5, 6) by a process incorporating a diversity (D_H) element (5). Transcripts of the rearranged V_H-D_H-J_H-C_μ gene (7) are spliced to remove the large intervening sequence between the J_H and C_μ regions and the three small intervening sequences that separate the four domains of the C_μ gene (3, 5, 8, 9). Further splicing can generate two functional μ mRNA species with different 3′ termini (10–12); a 2.4-kilobase (kb) species contains a "μ_S" segment encoding the carboxy terminus of the secreted form of μ chain, while a 2.7-kb species contains a "μ_M" segment specifying that of the membrane-bound form of μ chain, which mediates antigen-receptor function in B cells.

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Recently we found a different class of C_μ transcripts in T cells (13, 14). We had looked for expression of the C_μ gene to determine whether the heavy chain locus might contribute to the immunological specificity of T cells. A number of T lymphoma cell lines (13) and normal mouse thymocytes (14) contained multiple polyadenylylated RNA species bearing C_μ sequences (designated here as C_μ RNAs) with characteristic sizes distinct from those of B cell μ mRNAs. C_μ RNAs were also present in lines representing immature B cells (13, 15) but not in erythroleukemia, mastocytoma, or sarcoma cells. Surprisingly, they were present in myeloid cells (i.e., cells of granulocyte–macrophage lineage), which are not thought to express immunological diversity (13). Because the J_H locus was rearranged in only one of the C_μ RNA-expressing T and myeloid lines examined (3, 13), we proposed that the C_μ gene might be activated by a mechanism independent of J_H rearrangement (3). Although these results suggest that the heavy chain locus functions in T cells, serological studies on cell lines that contain C_μ RNA but not μ mRNA have failed to detect μ chains (I. D. Walker and A. W. Harris, personal communication).

We have investigated here the structure of the C_μ RNAs and have examined whether they express immunological diversity. By hybridization tests with fragments from a cloned plasmacytoma μ gene, we show that C_μ RNAs are equivalent to μ mRNAs throughout the C_μ region and that they possess either the μ_S or the μ_M 3′ region. However, C_μ RNAs differ markedly from μ mRNAs in their 5′ regions. A 5′ sequence element on two C_μ RNAs is encoded by a chromosomal segment located between the J_H and C_μ genes. The results suggest that C_μ RNA represents a distinct form of C_μ transcription characteristic of early stages of lymphoid development and that regulation of C_μ gene expression includes changes in splicing.

MATERIALS AND METHODS

Procedures for RNA extraction from cells, gel electrophoresis of denatured polyadenylylated RNA (16), transfer to diazobenzyloxymethyl paper (17), and hybridization with cloned DNA probes have been described (13, 14). Cloned cell lines grown *in vitro* were T lymphoma line STRij-4-2.2 (abbreviated ST4), B lymphoma lines WEHI-231.1. and WEHI-279.1 (W231 and W279), myeloid lines WEHI-265.1 and RAW8.2 (W265 and RAW8) (13), and Abelson pre-B lymphoma line 18-81 (18) kindly provided by E. J. Siden. Plasmacytoma HPC-76 (H76) was propagated in BALB/c mice. Cloned chromosomal DNA sequences Ch.H76μ1 (8) and Ch.H76μ119 (3, 6) and μ cDNA clone pH76μ17 (19) have been described. Probes were fragments isolated by polyacrylamide gel electrophoresis from di-

Abbreviations: V, variable region; J, joining region; C_μ, μ constant region; D, diversity region; H, heavy chain; kb, kilobase(s).

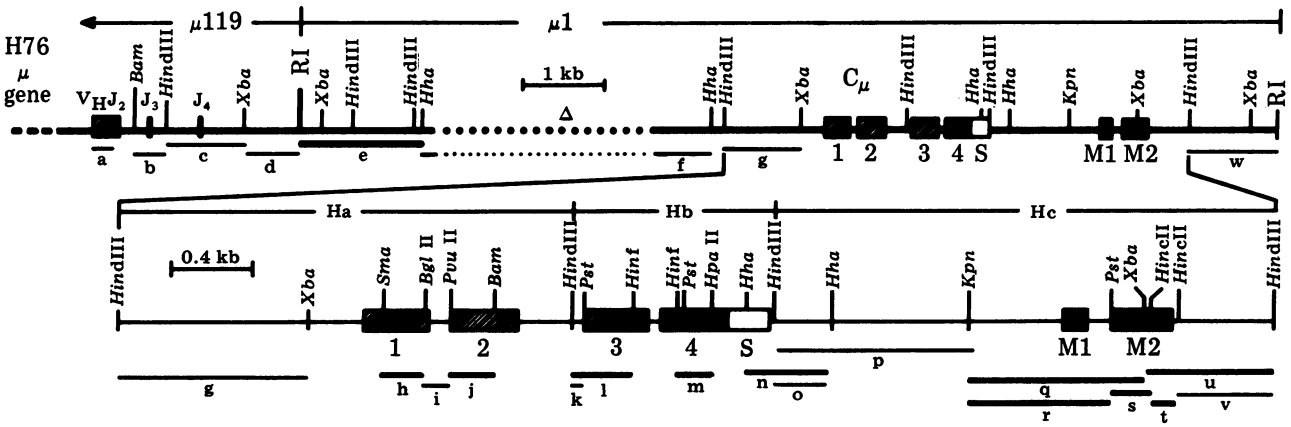

FIG. 1. Structure of a cloned plasmacytoma μ gene and derivation of fragments from it. Cloned *Eco*RI (RI) fragments μ1 and μ119 spanning the active V_H-J_H-C_μ gene of plasmacytoma H76 have been described in detail (3, 6, 8). The positions of the μ_S segment (S) and the μ_M segment (M1 + M2) shown here are based on correlating our restriction map with that of Early *et al.* (11). The dotted region (Δ) indicates a segment present in the germ line but deleted from the H76 genome (3). The lettered fragments indicated by heavy lines are those that hybridized to one or more species of C_μ RNA (see text), while those indicated by light lines failed to hybridize. The expanded map shows three *Hin*dIII fragments (Ha, Hb, Hc) subcloned in pBR322 by Ora Bernard. Fragments a–d were prepared from subclones of μ119; e, f, and n from a *Hha* I digest of μ1; w, from a *Hin*dIII/*Eco*RI subclone of μ1; and each of the others by the relevant digest of Ha, Hb, or Hc. Only the *Hin*f, *Pvu* II, *Hin*cII, and *Hpa* II sites relevant to the probes are shown.

gests of μ1, μ119, or subclones of them, as detailed in Fig. 1. Each was ^{32}P-labeled by nick translation (20).

RESULTS

To determine which sequences within or near the C_μ gene were present within individual C_μ RNA species, we fractionated RNA from representative cell lines by gel electrophoresis (16) and subjected blots of the gels (17) to hybridization with small DNA fragments from a cloned plasmacytoma μ gene (3, 6, 8). Fig. 1 shows that the 23 fragments (a–w) used as probes span the 11.5-kb cloned DNA sequence from the V_H and J_H genes through the four C_μ domains, and include segments specifying the 3′ portion of μ_S mRNA (S), and that of μ_M mRNA (M1 + M2). Autoradiographs in Fig. 2 display the hybridization to RNA from T lymphoma ST4 obtained with selected probes. We previously reported that ST4 contains C_μ RNA species of 1.9, 2.2, and 3.0 kb (13). In many subsequent experiments, the components of ≈2 kb have partially resolved into at least three species designated as 1.9-, 2.1-, and 2.3-kb C_μ RNAs.

Each C_μ RNA Contains the Four C_μ Domains but not the Intervening Sequences Separating Them. When fragments specific for each C_μ domain (h, j, l, and m in Fig. 1) were tested, each revealed a spectrum of C_μ RNA species indistinguishable from that given by the cDNA probe, as illustrated in tracks h and j of Fig. 2. Moreover, each domain probe labeled the C_μ RNAs to the same extent as it labeled the μ mRNAs of B lymphoma and plasmacytoma RNA controls, and similar results were obtained with myeloid line W265 (data not shown). Unlike the domain probes, fragments from the intervening sequences between domains 1 and 2 (fragment i) and between domains 2 and 3 (fragment k) did not hybridize to ST4 RNA (tracks i and k in Fig. 2). Sequences between domains 3 and 4 were not tested. Within the limitations of the technique, these results suggest that the splicing and hence the structure of C_μ RNAs throughout the C_μ region is similar or identical to that of μ mRNAs.

Each C_μ RNA Has Either a μ_M or a μ_S 3′ Terminus. We used fragments 3′ to domain 4 to identify the 3′ ends of C_μ RNAs. Fragment n, specific for μ_S, labeled only the 1.9-kb C_μ RNA (Fig. 2, track n). In contrast, a μ_M probe (fragment q) hybridized to the 2.1-, 2.3-, and 3.0-kb species but not to the 1.9-kb (track q). Sequences between S and M1 (fragments o and p) were not detectable on any species of ST4 (or W265) RNA (e.g., track p). Hence, like μ mRNAs, the C_μ RNAs have been processed in alternative ways at the 3′ terminus. Tests with probes r–v (Fig. 1) suggest that the μ_M termini include both exon M1 and exon M2 (data not shown). Fragment r, which includes M1 but not M2, hybridized to the 2.1-, 2.3-, and 3.0-kb species. Hence all three RNAs contain an element, presumably M1, from within the 700-base-pair sequence of this probe. Fragments s, t, and v define M2 more precisely, because s and t are located almost entirely within M2 and together span it, while v commences 50 bases 3′ to M2 (11). Because the hybridization patterns given by fragments s, t, and u were similar whereas fragment v failed to hybridize, the 2.1-, 2.3-, and 3.0-kb C_μ RNAs must each contain essentially all of M2 and must terminate (or be spliced) near or at its 3′ boundary.

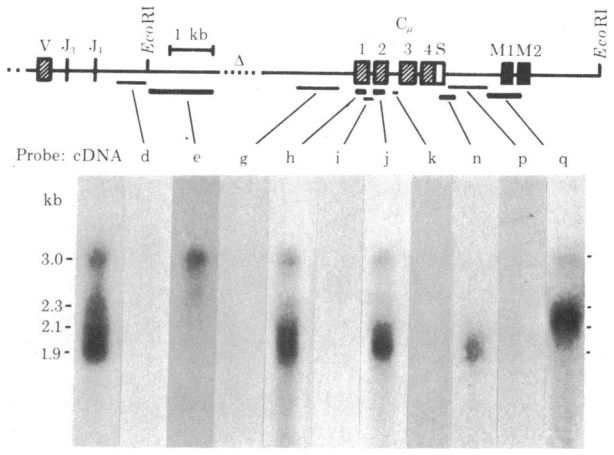

FIG. 2. Detection of sequence elements in C_μ RNAs of T lymphoma line ST4. For each track, 5 μg of polyadenylylated ST4 RNA was denatured with glyoxal, fractionated by gel electrophoresis, transferred to diazobenzyloxymethyl paper, hybridized with a [^{32}P]DNA probe, and autoradiographed, all as detailed elsewhere (13). For the "cDNA" track the probe was an *Mbo* II fragment spanning domains $C_{\mu 1}$ + $C_{\mu 2}$, from cDNA clone pH76μ17 (8, 19). For tracks d–q the probes were the corresponding restriction fragments defined in Fig. 1 and indicated on the map here. The autoradiographs were exposed either for 3 days (tracks k, i, and cDNA) or 5 days. The sizes of C_μ RNAs indicated to the left were determined by assuming mouse ribosomal 28S and 18S RNAs to be 4.7 and 2.0 kb long.

Because sequences from the cloned region extending 1.6 kb 3′ to M2 (v and w in Fig. 1) did not hybridize to any C_μ RNA species, we surmize that their poly(A) sequences are generated at the known poly(A)-addition sites at the end of μ_S and μ_M (10, 11). We conclude that the C_μ RNA species are indistinguishable from their μ mRNA counterparts from C_μ domain 1 to their 3′ termini. Thus we can account for 1.48 kb of the μ_S-bearing C_μ RNA and 1.68 kb of the μ_M-bearing C_μ RNA species [not including poly(A)].

A 5′ C_μ RNA Segment Mapping Between the J_H and C_μ Genes. On testing fragments spanning the region between the J_3 gene and domain 1 (b–g in Fig. 1), we were surprised to find hybridization with fragment e, which maps between the J_H and C_μ genes. Fig. 2 (track e) shows that it hybridized to a 3.0-kb species in ST4 RNA to an extent comparable to that obtained with any C_μ probe. We think that fragment e hybridized to the 3.0-kb C_μ RNA rather than an unrelated 3.0-kb species because only RNA from cell lines that contain C_μ RNAs hybridized and the extent correlated with the abundance of the 3.0-kb C_μ RNA in different lines. Fragment e also hybridized well to the 2.3-kb C_μ RNA of myeloid line RAW8 and possibly to that species in ST4 RNA (see below). Thus two C_μ RNA species contain sequences that map between 5.0 and 6.5 kb 5′ to the C_μ gene, within the conventional J_H-C_μ intervening sequence.

No significant hybridization to ST4 or W265 RNA was obtained with any other fragment 5′ to the C_μ gene (e.g., tracks d and g in Fig. 2). We conclude that no segment tested except fragment e is represented within the C_μ RNAs. However, a 2.7-kb germline segment within the region between e and $C_{\mu 1}$ (Δ in Figs. 1 and 2) could not be tested because it has been deleted from the H76 genome (3), and therefore is not present in the μ clone used here. In addition, sequences shorter than about 50 base pairs, such as J_H coding regions, would not have been detected.

C_μ RNA Species in Myeloid, Pre-B, and B Lymphoid Lines Are Equivalent to Those in T Cells. Fig. 3 shows the hybridization of probes specific for the C_μ gene and region e 5′ to the C_μ gene to RNA from a pre-B, a T, a myeloid, a B, and a plasmacytoma cell line. The C_μ probe revealed a marked similarity between the C_μ RNA patterns in the pre-B, T, and myeloid cell lines. Significantly, the probe from region e clearly labeled the 3.0-kb species in each of these lines and in the B lymphoma line as well. It also clearly labeled the 2.3-kb myeloid species. We attribute its labeling of the very abundant 2.4- and 2.7-kb B lymphoma components to a trace contamination of the probe with C_μ sequences.

Testing RNA from each line with the μ_S and μ_M probes revealed that each C_μ RNA species bears either a μ_S or μ_M terminus (data not shown). The pre-B line 18-81 contained, in addition to T-like species, a μ_M-bearing component of $\approx$2.7 kb and a μ_S-bearing component of $\approx$2.4 kb, like those in B cells. We therefore interpret its complex pattern (pB in Fig. 3) essentially as a combination of the T and B patterns. The presence of μ mRNAs in this line is expected, because it synthesizes μ chains (18). The myeloid line contained, in addition to the species that are prominent in ST4, a 2.8-kb component bearing μ_S. The pre-B line appeared to contain a similar component, which may represent a μ_S counterpart of the μ_M-terminated 3.0-kb species. The relative abundance of the different C_μ RNA species varies between different Abelson, T, and myeloid cell lines (ref. 13 and unpublished results) and also varies somewhat between RNA batches from a single line.

V_H Sequences Are Not Detectable on C_μ RNAs. In considering possible biological roles of C_μ RNAs, it is important to know whether they contain V_H regions. Experimental ap-

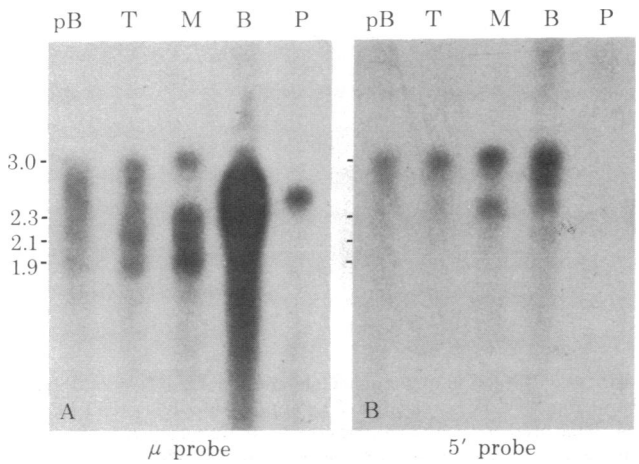

FIG. 3. Relationship of C_μ RNA species in cells of the T and B lymphoid and myeloid lineages. Experimental details were as in Fig. 2, except that the tracks contained 5 μg of polyadenylylated RNA from Abelson pre-B line 18-81 (pB), T lymphoma line ST4 (T), myeloid line RAW8 (M), B lymphoma line W231 (B), or 5 μg of unfractionated RNA from plasmacytoma H76 (P). In *A*, the probe was a C_μ probe ($C_{\mu 3}$ + $C_{\mu 4}$), whereas in *B* it was a fragment 5′ to the gene (fragment e in Fig. 1).

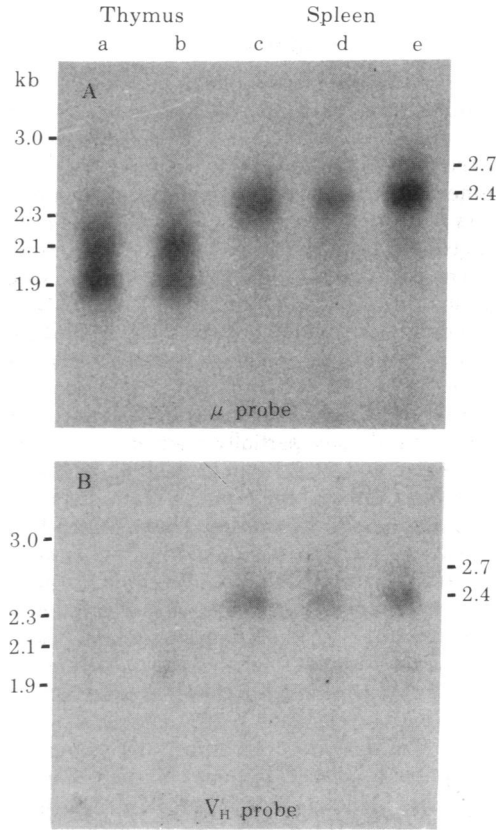

FIG. 4. RNA species of thymocytes and splenic B cells revealed by C_μ and V_H probes. (*A*) RNA from thymocytes and splenic B cells scored with a C_μ probe; (*B*) an identical filter scored with a V_H probe (fragment a, Fig. 1). Tracks a and b contained $\approx$5 μg of polyadenylylated RNA from thymocytes depleted of contaminating B cells by treatment with anti-Ia^k serum plus complement or from untreated thymocytes, respectively. Tracks c–e contained $\approx$0.5 μg of polyadenylylated RNA from spleen cells of the athymic *nu/nu* mouse, from spleen cells depleted of T cells by treatment with anti-Thy-1 serum plus complement, or from crude spleen cells, respectively. Autoradiography was for 2 days (*A*) or 10 days (*B*). Other details were as for Fig. 2.

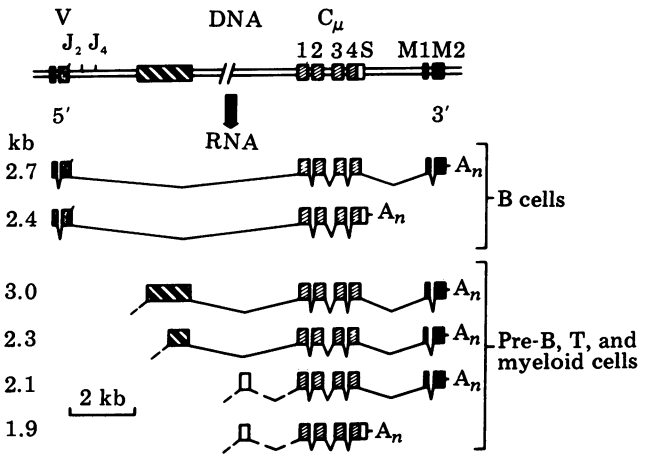

FIG. 5. Models for the structure and splicing of C_μ RNAs. The structure of the chromosomal μ gene shown at the top is that of plasmacytoma H76 (Fig. 1) and the relevant sequence elements are indicated by boxes. For each transcript, boxes (matching those for the chromosome) represent sequence elements present in the corresponding C_μ RNA or μ mRNA, while the lines connecting them represent intervening sequences removed by splicing from the presumptive precursors(s). Within the $C_{\mu 1}$-$C_{\mu 4}$ domain region, the splicings are shown as identical to those established for μ mRNAs (6, 8, 9). The splicings of the μ_S and μ_M regions of the μ mRNAs have been established by nucleotide sequence determination (10, 11). The lengths of 5' elements on the 3.0- and 2.3-kb C_μ RNA s are not known and are arbitrarily shown as the lengths required to bring each species to its estimated full size. Broken lines (and unfilled boxes) at the 5' ends of C_μ RNAs indicate that additional splicings of other small elements cannot be ruled out.

proaches are complicated by the fact that any particular cell clone would be expected to express only one of the several hundred different V_H genes, only a few of which cross-hybridize with a given V_H probe (2, 4, 21).

We have tested RNA from five T lymphoma and four myeloid lines that contain C_μ RNAs (13), using two distinctly different V_H probes (data not shown). The first (fragment a, Fig. 1) did not detect V_H regions in any of these lines, or in B lymphomas W231 and W279. The second, a cloned V_H region from plasmacytoma HOPC 1 (19), hybridized strongly to the 2.4- and 2.7-kb μ mRNAs of W279 and weakly to those of W231, but did not hybridize to any T or myeloid RNA. It is not possible to draw a firm conclusion from such a small sample of V_H probes.

As an alternative approach, we scored RNA from normal mouse thymocytes and from splenic B cells. In contrast to cloned cell lines, splenic B cells express a diverse array of V_H regions, and this might be expected also for thymic T cells. Fig. 4*A* shows that, as reported previously (14), C_μ RNAs are present in untreated thymocytes (track b) and in those treated with an anti-Ia serum (14) to deplete contaminating B cells (track a), while the distinctive 2.4- and 2.7-kb μ mRNAs are found in three different splenic B cell populations (tracks c–e). In contrast, Fig. 4*B* shows that a V_H probe (fragment a) failed to hybridize to the thymocyte C_μ RNAs (tracks a and b). The 2.4- and 2.7-kb μ mRNAs of B cells were labeled (tracks c–e), as was an RNA species of $\approx$2.0 kb, possibly δ and/or γ mRNA. A trace of the 2.0-kb species was present in untreated thymocytes (track b) but not in those depleted of contaminating B cells (track a). If the C_μ RNAs of thymocytes do contain V_H regions, their repertoire of V_H sequences must be distinctly different from that of B cells. However, even in the B cell tracks the V_H signal was low and we would not have detected V_H regions if they had been present on the less abundant 3.0-kb C_μ RNA species of thymocytes.

DISCUSSION

Transcripts of the C_μ Gene Are Spliced by Alternative 5' and 3' Pathways. From the results presented here, we infer that the distinctive class of C_μ transcripts present in T and pre-B lymphoid and myeloid cells are generated as shown in Fig. 5. Each C_μ RNA appears to have the same structure as μ mRNA throughout domains 1–4 and bears either the μ_S or μ_M 3' terminus found on μ mRNAs. In their 5' regions, C_μ RNAs differ markedly from μ mRNAs. One striking difference is that the 3.0- and 2.3-kb C_μ RNAs bear a 5' sequence element encoded within the conventional J_H-C_μ intervening sequence. Because this element is located 5.0–6.5 kb 5' to the C_μ gene, a large intervening sequence must have been removed by splicing. Thus at least one previously unsuspected splice site occurs within the J_H-C_μ region. The different lengths of C_μ RNAs must be generated by alternative splices in the 5' region, by different transcriptional starts, or both.

The C_μ RNA species do not appear to encode immunological diversity, because (*i*) we were unable to detect V_H sequences on them, (*ii*) the 1.9-, 2.1-, and 2.3-kb C_μ RNAs are several hundred bases shorter than the corresponding 2.4-kb μ_S and 2.7-kb μ_M mRNAs known to bear V_H sequences, and (*iii*) C_μ RNAs can be made by cell lines that have not undergone J_H rearrangement. No definite conclusion can, however, be reached until all their 5' terminal components have been accounted for.

The production of C_μ RNAs, unlike that of μ mRNAs, does not require a recombined J_H locus (3). Because this form of transcriptional activation of the C_μ gene is not dependent upon V_H-D_H-J_H joining, it may well represent an early event in the molecular pathway to expression of μ mRNAs. One possibility is that transcription always initiates at site(s) to the left of the J_H locus regardless of whether rearrangement has occurred. If so, C_μ RNAs and μ mRNAs might be viewed as the products of alternative 5' processing pathways of the precursor RNAs, with correct V_H-D_H-J_H rearrangement being required for the J_H-$C_{\mu 1}$ splicing that generates μ mRNAs. Alternatively, the transcriptional start(s) for C_μ RNAs may lie to the right of the J_H locus. Any model must account for the observation that the C_μ RNAs in lines that have undergone J_H rearrangement on both

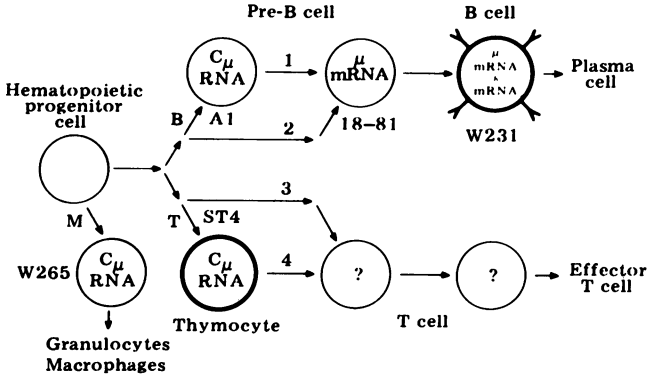

FIG. 6. Expression of the C_μ gene during development of cells of the B and T lymphoid and myeloid lineages. Tumor cells that represent stages of the developmental paths are indicated by circles with a cell line designation and stages studied both as tumors and as normal cells are indicated by heavy circles. Membrane-bound immunoglobulins are indicated for line W231. Expression of C_μ RNA or μ mRNA is shown within the cells, but when both were present, only the latter is indicated. Developmental paths 1 and 4 (in the B and T lineages, respectively) indicate the possibility that expression of C_μ RNA is an obligatory step in the molecular pathway leading to expression of the B and T antigen receptors, while paths 2 and 3 represent C_μ RNA expression as a nonessential developmental event.

alleles are the same size as those in lines in which no J_H rearrangement has occurred (3). An analogous transcription of the C_κ gene on unrearranged chromosomes occurs in many plasmacytomas, but the C_κ transcripts (8.3 kb) do not undergo splicing (22).

Biological Implications of C_μ RNA Expression in T, B, and Myeloid Cells. The cells in which C_μ RNAs have been detected are thought to represent early stages of development of the B, T, and myeloid lineages (Fig. 6). Thus Abelson lymphoma lines represent B cells at stages prior to expression of surface immunoglobulin as an antigen receptor (18), whereas the bulk of normal thymocytes (23) and most T lymphomas represent immature T cells and the myeloid tumor lines represent precursors of granulocytes and macrophages (13). C_μ RNA expression may therefore reflect an early developmental stage of C_μ gene activation, perhaps initiated in a cell that is a progenitor of the T, B, and myeloid lineages.

In the B lineage C_μ RNA expression is, on one model, an obligatory early step in the molecular pathway leading to expression of immunological diversity (path 1 in Fig. 6). Correct V_H-J_H joining at a later stage of development is necessary for production of μ mRNA. In an alternative model, C_μ RNA synthesis reflects instead an abortive form of C_μ gene activation, and cells in which that form of activation occurs on both homologous chromosomes would be committed to a dead-end pathway rather than the pathway (2 in Fig. 6) leading to immune competence. Pre-B and B lines that contain both C_μ RNAs and μ mRNAs, such as 18-81 and W231, indicate that both modes of transcription and splicing can occur in the same cell line, although it is not clear whether both originate on the same chromosome. The C_μ RNA synthesis in such lines might reflect "allelic exclusion" (1), whereby only one chromosomal complement is expressed as immunoglobulin.

Our results demonstrate that early events in expression of the heavy chain locus in the B lineage have a counterpart in the T lineage, because the C_μ RNAs of T lymphoma cells and thymocytes are equivalent to those of pre-B lymphoma cells and J_H rearrangement can occur in both lineages (3). It is not yet clear, however, whether C_μ RNA expression in T cells is a necessary step towards expression of immunological recognition (path 4 in Fig. 6) or whether an independent path (path 3) leads to immunocompetence.

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