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TITLE OF STUDY (*DO NOT EXCEED 70 CHARACTERS*)
Function of Surrogate Light Chains In B Cell Development

KEY WORDS (LIMIT TO FIVE)
B cell development, mu chain, pre-B cell receptor

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NA-FACE.DOC 08/13/93

1. a. P.I. : **Hans-Martin Jäck**
   b. Institution : **Loyola University Chicago**
   c. Title of Project : **Function of Surrogate Light Chains in B Cell Development**

d. Clearly summarize BACKGROUND, WORKING HYPOTHESIS and BROAD GOALS of the project. Do not use additional pages

As a long-term goal we wish to understand how mature antigen-responsive B cells develop from pluripotent stem cells in the bone marrow of mice. In this proposal, we will focus on the role immunoglobulin (Ig) and Ig-like polypeptides play during the transition from Ig-negative pro-B cells to Ig-positive pre-B and B cells.

B cells express IgM receptors that consist of two heavy (H) chains (μ chains) and two light (L) chains, whereas pre-B cells, the precursor of IgM-positive B cells, express μ chains and surrogate light (SL) chains, but not conventional L chains. SL chains, which are composed of the two non-covalently associated polypeptides, VpreB and λ5, form with the membrane form of μ chains a disulfide-linked tetrameric Ig-like complex (reviewed in 1). Although the function of this complex is still unknown, knockout experiments in mice have shown that the membrane form of μ (μ_m) as well as λ5 play an important role in the control of B cell maturation (reviewed in 2, 13). For example, B cell development is blocked in these mice at the large $B220^+$, $CD43^+$, IgM^- pre-B cell stage, which results in a depletion of small $B220^+$, $CD43^-$, IgM- pre-B cells. From these data, it was hypothesized that only large pre-B cells that can assemble a μ/SL complex proceed to develop into mature B cells. If this is true, then we expect to find in mature B cells only μ chains that were able to form a complex with SL chains in the large pre-B cell stage. But in the large pre-B cell population we may well find cells expressing μ chains that do not form a complex with SL chains. Such pre-B cells would, however, be inhibited from differentiating into mature B cells. We have recently identified pre-B cell lines with μ chains that do not assemble with SL chains (3). The Ig variable (V) region that is utilized by these pre-B cells is the V_H81X region, a V region that although very frequently expressed in pre-B cells (4) is rarely detected in mature B cells (4, 5). From these data we proposed that V_H81X/μ pre-B cells do not differentiate into mature B cells because they cannot assemble a μ/SL complex. In our AIM 1, we will use the V_H81X system to test this idea by analyzing the B cell population in the bone marrow of a transgenic mouse that expresses only V_H81X/μ chains.

One function of the μ/SL complex might be that it triggers the differentiation signal either from a compartment within the cell or from the cell surface after its interaction with a putative extracellular ligand (6). That this complex can be detected on the surface of transformed pre-B cell lines (reviewed in 1) and normal human pre-B cells (7) and that μ/SL complexes have signal-transducing capability (8, 9, 10) supports the surface signal hypothesis. However, μ/SL complexes cannot be detected on the surface of normal bone marrow cells of mice, which supports the idea that the receptor can signal from within the cell. In our AIM 2, we will distinguish these two possibilities by analyzing a transgenic mouse that assembles but does not transport the μ/SL complex to the cell surface.

Not much is known about the mechanisms by which the expression of SL chains is controlled during B cell development. We know that expression of λ5 and VpreB is terminated during the transition from a pre-B cell into a IgM-positive B cell (11, 12) and preliminary results from our lab indicate that this down-regulation is a direct result of the expression of IgM on the surface of the B cell. In our AIM 3, we will further investigate the mechanism by which IgM downregulates the expression of λ5 in pre-B cell lines.

2. List the **SPECIFIC AIMS** of this research project (as opposed to the broad goals presented in Item 1). Do not exceed the space allotted below. and k

AIM 1: Determine whether a transgenic mouse that only expresses transgenic V_H81X/μ chains develops mature B cells

Analyze the B cell population in a V_H81X/μ transgenic mouse by flow cytometry

AIM 2: Determine the mechanism by which IgM terminates λ5 expression

- **a.** Confirm that IgM expression downregulates λ5 expression by analyzing λ5 expression in μ-positive pre-B cell lines before and after the induction of a transfected κ gene
- **b.** Determine which form of IgM is required to downregulate λ5 expression by analyzing λ5 expression in pre-B cells that express either the membrane or the secreted form of μ chains
- **c.** Determine whether surface expression of IgM is required to downregulate λ5 by analyzing λ5 expression in a pre-B cell line that expresses μ-TRAP (see AIM 3a) and κ
- **d.** Identify by DNA gel-shift assays proteins that bind to the λ5 promoter region in an IgM^-- and an IgM^+ -pre-B cell line

AIM 3: Determine whether μ/SL complexes are required on the surface of pre-B cells to trigger them to develop into B cells

- **a.** Engineer a gene that encodes μ chains with an ER-retention signal and test in a pre-B cell line whether this modified μ chain (μ-TRAP) is retained in the ER and induces K gene rearrangement
- **b.** Generate a transgenic mouse that expresses μ-TRAP (μ-TRAP mouse)
- **c.** Analyze the B cell population in a μ-TRAP mouse by flow cytometry

3. **SUPPORTING DATA, EXPERIMENTAL DESIGN and PROCEDURES.** Do not attach more than six (6) additional pages (3a-3f). All figures, charts, tables, and references must fit within pages 3 - 3f.

A. SUPPORTING DATA

In this chapter, I will briefly discuss our two major findings that motivated us to develop this grant application.

Identification of a μ chain that does not associate with SL chains.

To determine in vivo whether the assembly of a μ/SL complex is a prerequisite for large pre-B cells to differentiate into small pre-B and B cells (AIM 1), we need an H chain that does not bind to SL chains. One candidate for such an H chain might be a μ chain with the V_H81X region, because V_H81X regions, although they are very frequently utilized in pre-B cells (reviewed in 4), are rarely used in mature B cells (4, 5). Indeed, when we analyzed immunoprecipitated [^{35}S] methionine-labeled proteins in the Abelson virus-transformed V_H81X/μ-positive pre-B cell line F (14) by SDS-PAGE, we detected under reducing conditions in anti-μ precipitates μ chains and VpreB, but not λ5 (Fig. 1b, lane 4). λ5, however, could easily be detected in anti-λ5 precipitated material of F cells (Fig.1b, lane 5). When we analyzed the same immunoprecipitates under nonreducing conditions, μ-containing complexes (labeled with "μ" and "μ_2") were only detectable in anti-μ (Fig. 1c, lane 2) but not in anti-λ5 precipitated material (lane 3) of F cells. Similar results were obtained by analyzing another V_H81X-positive pre-B cell line (BFL 23, ref. 15, Fig. 1C, lanes 6 and 7). In contrast, we detected, as expected, monomeric μ chains under reducing (Fig.1b) and μ-containing complexes under nonreducing conditions (Fig. 1c) in both anti-μ and anti-λ5-precipitated material of surface μ-positive pre-B cell lines (107.2 and 2G4). We confirmed these findings in pre-B cells that were transfected with either a V_H81X/μ gene or a V_HJ558/μ gene (data not shown). We conclude from these results that V_H81X regions prevent the covalent association of μ chains with λ5. complexes. We speculate further that large pre-B cells

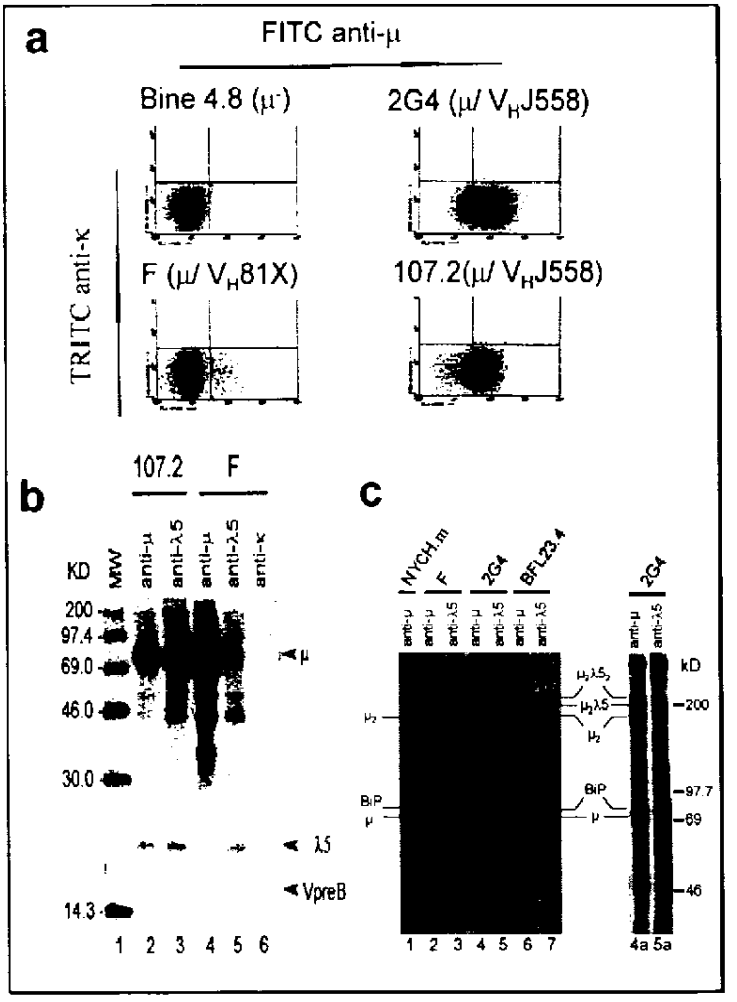

Fig. 1: Analysis of μ and SL expression in pre-B cell lines. (a) Cells were stained with a mix of a FITC-labeled anti-μ and RITC-labeled anti-κ antibodies and analyzed by flow cytometry. The μ-negative pre-B cell line, Bine 4.8, served as a negative control and 2G4, which was generated by transfecting Bine 4.8 with a μ gene containing a V_HJ558 region, as a positive control. 107.2 is a surface positive pre-B cell line. **(b,c)** Equal aliquots of [^{35}S] methionine-labeled cell extracts were immunoprecipitated with monoclonal anti-μ, anti-κ, and anti-λ5 antibodies, followed by S. aureus. Solubilized proteins were resolved on **(b)** a reducing 12.5% and **(c)** a nonreducing 7.5% SDS polyacrylamide gel, and detected by fluorography. The 16kD is very likely VpreB, because it has the predicted MW of and co-precipitates with μ as well as with λ5 chains. NYCH.μ is a μ-only hybridoma cell line (see manuscript in Appendix)

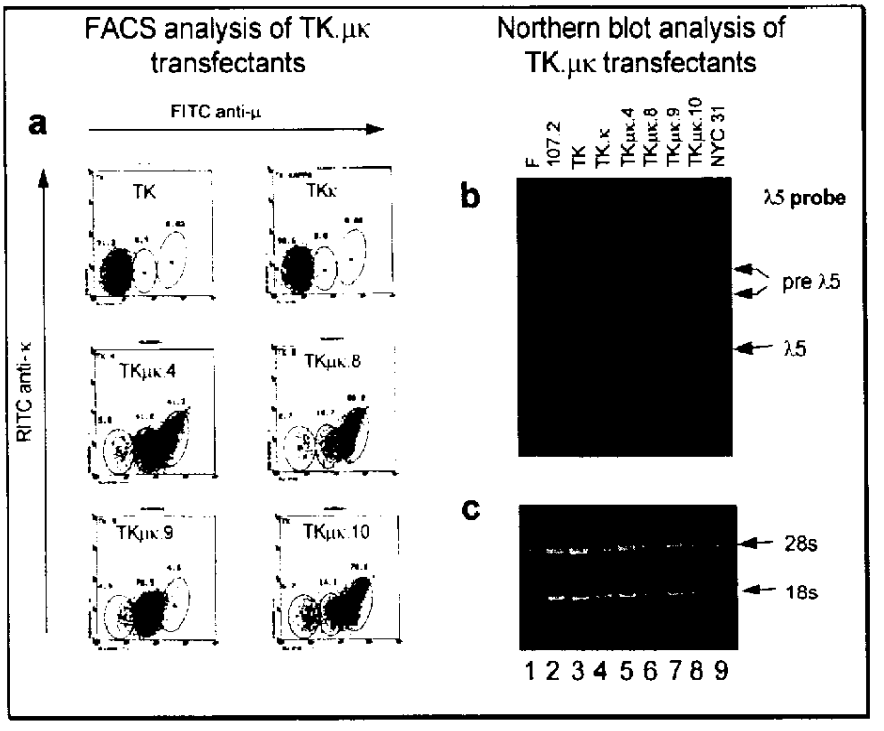

Fig. 2: Analysis of TK.μk cultures a) Cells were stained and analyzed by FACS as described in Fig. 1a. The same windows for Ig-negative, μ-positive and μ,k-positive cells were set in all sorts. **b, c)** 5μg of RNA was separated on a 1.2% HCHO/MOPS gel and transferred onto nitrocellulose. **b.** The filter was hybridized with a [^{32}P]-labeled λ5 probe and bands were detected by autoradiography. **c.** Photograph of the EtBr-stained gel before the transfer. NYC31 is a λ5$^-$ B cell line.

that express these μ chains do not receive a positive signal and, therefore, do not proceed to develop into small pre-B and subsequently into mature B cells.

Since V_H81X/μ chains do not associate with λ5 chains, these H chains might still be trapped in the ER by the heavy chain binding protein, BiP (24). Indeed, we did not detect μ chains on the surface of V_H81X pre-B cell lines F (Fig. 1a) and BFL23 (data not shown). In contrast, μ chains with a V_H region different from V_H81X (e.g., V_HJ558) could easily be detected on the cell surface (107.2, 2G4). Taken together these results indicate that V_H81X regions prevent the assembly of μ chains with λ5 and, as a consequence, these μ chains are trapped within the cell, presumably by the molecular chaperone BiP. We, therefore, think that a transgenic mouse that expresses only V_H81X/μ chain might be a perfect model to address the question of whether the binding of μ chains with SL chains is a prerequisite for pre-B cells to develop into B cells (AIM 1).

Co-expression of μ and κ correlates with downregulation λ5 in the pre-B cell line TK

To test our idea that surface IgM receptors downregulate the expression of λ5, we compared in various IgM-positive pre-B cell clones (TK.μk clones) the frequency of surface IgM-positive cells (Fig.2a) to levels of λ5 mRNA (Fig. 2b, c). For example, we detected in a TKμk.8 culture that contains about 80% surface IgM-positive cells (Fig. 2a) low amounts of λ5 mRNA (Fig. 2b, lane 6). On the other hand, in a Tkμκ.9 culture that contains about 5% IgM-positive cells we detected as much λ5 mRNA (Fig.2b, lane 7) as in TK cells that express only κ chains (lane 4) or no Ig chains at all (lane 3). From these preliminary data, we suggest that the downregulation of λ5 in IgM-positive TK clones is a direct result of the expression of surface IgM. This makes TK transfectants a perfect system to address the mechanisms by which IgM downregulates λ5 mRNA in transitional pre-B/B cells as suggested in AIM 2.

B. EXPERIMENTAL DESIGN

In this proposal, we will address three questions. First, is the assemble of a μ/SL complex a pre-requisite for large pre-B cells to proceed along the B cell differentiation pathway? Second, are μ/SL complexes required on the cell surface of pre-B cells to trigger a differentiation signal in these cells? And finally, do surface IgM receptors downregulate the expression of SL chains in transitional pre-B/B cells?

AIM 1: Determine whether a transgenic V_H81X/μ mouse develops mature B

We will address the first question by analyzing the B cell population in bone marrow of a transgenic mouse that expresses only V_H81X/μ chains. These H chains, as we have discussed earlier, do not assemble with λ5. Therefore, if the formation of a μ/SL complex is required in the B cell maturation pathway, we expect to find in a transgenic V_H81X/μ mouse the same effect on B cell development in the bone marrow as in mice that lack the membrane form of μ (μm) or λ5, that is, a developmental block at the stage of large $B220^+$, $CD43^+$, IgM^- pre-B cells and a depletion in small $B220^+$, $CD43^-$, IgM^- pre-B cells (reviewed in 2 and 13). Although both μm and λ5 knock-out have the same effect on early B cell development in the bone marrow, λ5 knock-out mice, in contrast to μm knock-out mice, have peripheral $B220^+/IgM^+$ B cells (13). These B cells might have been generated via the minor, or alternative B cell differentiation pathway, that is, from pre-B cells that rearrange, albeit with a very low rate, the κ chain locus in the absence of λ5 chains (2, 13). Although V_H81X/μ chains do not associate with λ5, they might associate with some κ chains to form IgM receptors. Therefore, we might find in a transgenic V_H81X some surface IgM $(sIgM)^+$ peripheral B cells. However, because not all κ chains might associate with V_H81X/μ chains (16), $sIgM^-$, cytoplasmic μ^+k^+ ($C\mu\kappa^+$) B cells might accumulate in the small $B220^+$, $CD43^-$, $sIgM^-$ pre-B cell population.

This will be assessed by determining the frequency of $B220^+/CD43^+/IgM^-$ (pro-B and large pre-B cells), $B220^+/CD43^-/IgM^-$ (small pre-B), and $B220^+/CD43^-/IgM^+$ (immature and mature B cells) in the bone marrow of transgenic and non-transgenic littermates by flow cytometry. Fortunately, Dr. John Kearney has already generated a transgenic V_H81X/μ (allotype μ^b) mouse line and agreed to provide the animals for our studies (letter of intend is included in the Appendix). In the next two chapters, I will first discuss Dr. Kearney's analysis of the splenic B cell population in his transgenic mouse. Then I will describe how we will analyze the bone marrow B cell population in the transgenic V_H81X mouse.

a. Analysis of the splenic B cell population in transgenic V_H81X mouse

Dr. John Kearney has presented the data of such an analysis at a recent B cell meeting in Montreal (his abstract in included in the Appendix). As predicted, the V_H81X/μ mouse showed a $\lambda5$ knock-out phenotype in the spleen, that is, older V_H81X/μ mice have normal numbers of splenic IgM^+ B cells, all of which expressed exclusively the transgenic μ chain, indicating that a μ/SL complex might not be required for allelic exclusion. In addition, the splenic B cells utilized just a few V regions in their κ chains. For example, 30% of V_H81X/IgM-positive spleen cell hybridomas used the same Vκ region in their k chains. One explanation is that V_H81X/μ chains preferentially pair with these κ chains and, therefore, only B cells that express these L chains are permitted to leave the bone marrow and populate peripheral lymphatic organs. In this case, the majority of B cells generated via the alternative pathway should not express surface IgM and accumulate in the small $B220^+$, $CD43^-$, $sIgM^-$ pre-B cell population. This will be assessed as described in the next paragraph.

b. Analyze B precursor populations in bone marrow of transgenic V_H81X mice

To analyze the B cell population in the bone marrow of transgenic and non-transgenic littermates, we will basically use the same approach others employed to investigate μ and $\lambda5$ knock-out mice (reviewed in 2 and 13) as well as normal mice (12). We will stain one aliquot of bone marrow suspension cells with fluorochrome-labeled anti-CD43, anti-B220, and anti-μ antibodies and another aliquot with isotype-matched fluorochrome-labeled control antibodies and analyze cells by three-color flow cytometry. CD43 is only expressed on pro-B and early, large pre-B cells, whereas B220 is a B cell marker that is expressed from the pro-B to the mature B cell stage (12). In addition, we will analyze the $B220^+/sIgM^-$ population by forward scatter to determine the frequency of small pre-B and large pre-B and pro-B cells in the bone marrow. If we find a lower number of small $CD43^-$, $B220^+/sIgM^-$ (small pre-B and $C\mu k^+$ B cells) in transgenic VH81X/μ animals than in non-transgenic animals, we conclude that this is because V_H81X/μ chains do not associate with $\lambda5$ chains. This would then be the first experimental evidence that only pre-B cells that assemble a μ/SL complex are permitted to proceed in the B cell differentiation pathway.

To determine the frequency of $sIgM^-$, $C\mu\kappa^+$ B cells in the bone marrow of transgenic and non-transgenic animals, we will sort about $5x10^4$ $B220^+/CD43^-/sIgM^-$ pre-B cells. This fraction should contain $C\mu k^+$ B cells and $C\mu^+$ pre-B cells. The purity of the sorted cells will be assessed by reanalyzing a small aliquot of the sorted population in the FACS. The sorted cells will then be fixed, stained with a mix of anti-μ and anti-κ or a mix of anti-μ and anti-λ antibodies and analyzed under the fluorescence microscope. This analysis will determine the frequency of $C\mu k^+$ and $C\mu\lambda^+$ B cells in the pre-B cell. If it is true that V_H81X/μ preferentially bind only to some L chains, then we expect a higher number of $C\mu k^+$, or $C\mu\lambda^+$ cells in a V_H81X/μ mouse than in a non-transgenic litter mouse.

AIM 2: Determine whether IgM terminates $\lambda5$ expression

The analysis of several μ,κ-transfected pre-B cell clones revealed that the level of $\lambda5$ mRNA correlates inversely with the frequency of IgM-positive cells in pre-B cell cultures (Fig. 2). From

these results we speculate that IgM signals a cell to downregulate the expression of λ5. As our immediate goals, we will first verify our initial observations and then determine whether the membrane or secreted form of IgM and whether IgM surface expression are required to downregulate λ5 expression. Finally, we will determine whether the regulation of λ5 expression occurs at the transcriptional or postranscriptional level and identify in IgM^+ and IgM^- pre-B cells proteins that bind to the λ5 promoter.

a. Verify that pre-B cells that express IgM downregulate λ5 expression

To verify our initial observations (Fig. 2) we will introduce an inducible κ gene into TK.μ transfectants that express μ chains on the cell surface (data not shown). Stable transfectants will be established and the expression of μ and κ analyzed by SDS-PAGE of immunoprecipitated Ig proteins. We will determine the frequency of surface IgM-positive cells by flow cytometry and the levels of λ5 mRNA by Northern blot analysis before and after induction of the inducible κ gene. If we find that surface expression of IgM correlates inversely with λ5 expression, we conclude that IgM directly is involved in the downregulation of λ5 expression. We will first use an improved metallothionein (MT) promoter that was recently described by Makarov and coworkers (23). This modified MT promoter has a low background activity in the absence and a high activity in the presence of Zn^{2+}. In case this system does not work satisfactory in stably transfected B cells, we will use the more time-consuming but very efficient tetracycline-controlled expression system (22) that has been successfully used in our lab to induce the expression of μ chains in stable transfected Hela cells (data not shown).

b. Determine whether the secreted or the membrane form of μ is required to downregulate λ5 expression

To determine whether the membrane or secreted form of IgM is required to terminate λ5 expression, we will first transfect a μ gene that encodes either the membrane or the secreted form of μ into the κ-positive cell line, TK.κ (Fig. 2) and then determine in stable transfectants λ5 mRNA levels by Northern blot analysis and IgM levels by flow cytometry and Western blot analyses. To generate vectors that allow the expression of either μ chain, we will delete the membrane exons or remove the poly(A) site of the μs mRNA from our prototype vector pμgpt (see manuscript in the Appendix), respectively.

c. Determine whether IgM is required on the cell surface to downregulate λ5 expression.

Membrane IgM might trigger a signal to downregulate λ5 from either inside the cell or after it has been transported to the cell surface. To distinguish these two possibilities, we will stably express in TK.κ a modified μ chain (μ-TRAP) that is trapped in the endoplasmic reticulum (ER). If we detect decreased levels of λ5 mRNA in μ-TRAP transfectants, then we conclude that IgM receptors can signal from inside the cell to downregulate λ5 expression. μ.TRAP will be generated by pasting the cytoplasm retention signal of calnexin, an ER-resident molecular chaperone (17) together with the cytoplasmic tail of μm chains. This will be accomplished by PCR using primers that contain the retention sequence of calnexin and sequences of the last μ membrane exon. The μ-TRAP gene will then transfected into TK.k cells and μ-TRAP as well as λ5 mRNA expression will be analyzed by SDS-PAGE, Western, and flow cytometry.

One major caveat of this approach might be that the modified μ-TRAP has lost its signal capability, presumably because sterical hindrance does not allow the association with signal transducing molecules, such as mb-1 and B29. We know that cross-linking of surface IgM with anti-μ antibodies increases intracellular Ca^{2+} levels in IgM-positive TK.μκ clones (data not shown), which suggests that wildtype IgM receptors in TK cells can associate with signal transducing molecules. We will perform two independent experiments to account for this problem. First, we will determine whether μ-TRAP still associates with mb-1 and B29. This will be accomplished by analyzing

proteins that were immunoprecipitated with anti-μ antibodies from digitonin lysates. We expect to find, in addition to μ, λ5, and VpreB, at least two additional proteins with MWs of approximately 32 and 36kD (18, 19). Second, we will determine whether μ-TRAP can induce κ gene rearrangement in a μ-negative subclone of the pre-B cell line, 300-19. Expression of μm chains, but not μs chains, in 300-19 cells induces rearrangement of the endogenous κ gene locus (20). If we detect in μ-TRAP-transfected 300-19 cultures the same frequency of κ-producing cells than in wildtype μm-transfected 300-19 cells (which will be assessed by cytoplasmic immunofluorescence), then we conclude that μ-TRAP chains can signal from within the cell to induce L chain rearrangement. We will then transfect TK.κ cells and determine in stable transfectants levels of λ5 mRNA by Northern blot analysis. Transfection with wildtype μm genes μ genes that contain a mutated form of the cytoplasm calnexin retention signal (we will mutate the three arginine and the one lysine residue in the retention signal to glycine residues, personal communication, Dr. T. Gallagher. Loyola University), will serve as positive controls and transfection with of μs genes as a negative control.

d. Determine whether the expression of λ5 is regulated at the transcriptional or the post-transcriptional level

Next, experiments will be performed to determine whether the downregulation of λ5 expression is a consequence of turning off the transcription of the λ5 gene or enhancing the turnover rate of λ5 mRNA. I have performed similar analyses to determine the turn-over rate of μ mRNA during B cell differentiation (see manuscript in the Appendix). In case, we find that the transcription rate of the λ5 gene decreases in IgM^+ TK cells, we will identify proteins that bind the λ5 promoter region (a gift from Dr. Bonnie Blomberg) by performing DNA gel retardation assays with nuclear extracts from IgM^+-TK and IgM^--TK transfectants.

AIM 3: Determine whether μ/SL complexes are required on the surface of pre-B cells to trigger them to develop into B cells

If we find in AIM 2 that μ-TRAP can activate κ gene rearrangement in the transfected pre-B cell line 300-19, we will use this construct to generate a transgenic μ-TRAP mouse. If surface expression of the membrane form of μ is a prerequisite for precursor B cells to develop into mature B cells, we expect the same phenotype as in a mouse that does not express μm chains at all (reviewed in 2). For example, precursors in the μ-TRAP mouse should do not develop past the large $CD43^+$ pre-B cell stage and they should lack small $B220^+$, $CD43^-$ pre-B/B cells in the bone marrow as well as $B220^+$ B cells in peripheral lymphatic organs. This can easily be determined by three-color flow cytometry using fluorochrome-labeled antibodies against B220, μ, and CD43.

A transgenic μ-TRAP mouse will be generated by the Transgenic Facility at Loyola University by introducing the μ-TRAP gene (allotype μ^b) into the germline of a mouse that contains endogenous μ gene segments of allotype **a**. Transgenic and non-transgenic littermates will be analyzed as described in AIM 1. In addition, we will analyze surface as well as cytoplasmic expression of both μ allotypes in bone marrow as well as peripheral (spleen and lymph nodes) $B220^+$ B cells. If we find in the bone marrow normal numbers of large $CD43^+$, small $CD43^-$ pre-B cells, and $C\mu k^+$ B cells, then we conclude that a μ/SL complex can trigger the differentiation signal from inside the cell. If we do not find any small $CD43^-$ pre-B cells, we conclude that μ-TRAP signals allelic exclusion of the endogenous μ (allotype b) genes from inside the cell. If we find normal numbers of $sIgM^+$ B cells and detect that they all have rearranged their endogenous μ genes, we conclude that μ chains have to reach the cell surface to signal pre-B cells to proceed in the B cell differentiation pathway and to exclude the rearrangements of μ genes. However, another possibility might be that μ-TRAP cannot signal from within the cell, despite the fact that it might activate κ gene rearrangement in a cell line (see AIM 2). If the endogenous μ genes in our μ-TRAP mouse are not allelically excluded, we will generate a μ-TRAP mouse that has lost its ability to generate endogenous μ genes by crossing the transgenic μ-TRAP gene into a homozy-

gous J_H knock-out mouse (21). The J_H knock-out mouse will be provided by Dr. Dennis Huszar (Genpharm, letter is included in the Appendix).

C. PROCEDURES

Recombinant DNA technology (DNA sequencing, plasmid cloning, PCR, site-directed mutagenesis, Southern and Northern blot analysis, e.t.c.), **mammalian cell culture, transfection of mammalian cells, metabolic labeling and immunoprecipitation of proteins and their analysis by Laemmli SDS-PAGE, two-dimensional PAGE and Western blot** are routinely used in my laboratory and documented in the my bibliography (see PI's CV and preprints in the Appendix). In addition, most of the constructs that will be used in this study are already finished. All **fluorochrome-labeled antibodies** are available and have been tested on bone marrow suspension cells from normal mice by **flow cytometry**, which was performed by Dr. Tom Ellis, Director of the Flow Facility at Loyola University (letter is included in the appendix). The **transgenic V_H81X mouse** will be provided by Dr. J. Kearney, University of Alabama (letter of intent in the Appendix), and the **transgenic μ-TRAP** mouse will be generated by Dr. Katherine Knight, Director of the Transgenic Faculty at Loyola University (a letter is included in the Appendix). Furthermore, Dr. Bonnie Blomberg, University of Miami, who has extensive experience in analyzing mammalian transcription will assist us in **DNA gel-shift assays** (letter is included in the Appendix). Therefore, I do not foresee any technical problems in performing the experiments suggested in this application.

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4. LABORATORY SPACE and FACILITIES available to the applicant.
State location if facilities are elsewhere than the applicant's institution.
Indicate any facilities that are required but are not currently available.

Laboratory Space: Dr. Jäck has a new, fully equipped laboratory of approximately 750 sq ft and a tissue culture room of approximately 100 sq ft.

Institutional Facilities:
Fluorescence Cell Sorter
Transgenic Facility
Macromolecular Analysis Facility
NIH Accredited Animal Facility

5. BUDGET JUSTIFICATION - Use this space to explain specific needs for items described on budget pages.

Animal costs are for generating transgenic mice as well as maintaining the VH81X transgenic mice at Loyola.

Because our cell lines grow only in the presence of 10% fetal calf serum (FCS) we need at least one liter of FCS per month. The plasticware is needed for all the tissue culture work.

Restriction enzymes, other enzymes, as well as primers are needed for generating expression vectors.

Isotopes are needed for labeling DNA proteins.

The fluorescence cell sorter is needed to analyze the B cell population in transgenic mice.

6. APPENDIX: Place the appendix materials after the original and each copy of the application form as indicated in the Instructions for New Applications.
   a. Biographical Sketches of the professional personnel to be associated with the project.
      Each sketch should be NO MORE THAN TWO (2) PAGES. The NIH format is acceptable.
      The P.I. should include and indicate by an asterisk the FIVE (5) most significant publications whether or not they relate directly to this application.
   b. Supporting material (such as letters of collaboration).
   c. Copies of not more than FIVE (5) of the applicant's publications or manuscripts that are pertinent to the project.

7. ABSTRACTS of PUBLICATIONS : *Only one set is required.* See Instructions for New Applications.
   Submit ONE PHOTOCOPY of the abstract page of each "pertinent publication" included in the appendix (6.c.) above;
   For each manuscript, submit a single composite page that includes authors, title, journal, abstract and publication status (for example, "submitted for publication").

P.I. Name Hans-Martin Jäck

Telephone Number 708-216-5816 **Grant Number** 4224

Budget rec H Hill 6/13

7. REVISED BUDGET (*based on award amount*):

A. Salaries. Give % time even if no salary is requested. State names or "to be recruited".

	% time	Amount
Professional Personnel including Principal Investigator		
Hans-Martin Jack, Ph.D	20	0
Technical Support		
Gabriele Beck-Engeser	25	7,573
Fringes @ 22.11%		1,674
A. Salaries Subtotal		9,247

B. Consumable supplies (by major category)

B. Consumables Subtotal 17,000

C. Other Expenses (itemize)

C. Other Expenses Subtotal 30,275

A+B+C Subtotal 56,527 56,522

D. INDIRECT COSTS (**15% of A + B + C**). D. INDIRECT COSTS 8,478

E. Permanent Equipment (itemize)

Permanent Equipment Subtotal 0

F. **TOTAL REQUEST** $65,000

REVISED

8. **Budget Summaries**

These buget amounts should reflect only modest changes to your current award.

BUDGET PERIOD	Salaries, Supplies and Other expenses	Permanent Equipment *	Indirect Costs	TOTAL
Year 2 if applicable	59,348	0	8,902	68,250
Year 3 if applicable	62,315	- 0 -	9,347	71,662

*You may not use CTR funds to purchase permanent equipment in the terminal grant year.

P.I. Signature Hans-Martin Jäck Fiscal Officer Signature

Date Typed Name of Fiscal Officer

REVISEDBUDOC rev9/10/93

7. FIRST YEAR'S BUDGET: P.I. Name Hans-Martin Jäck

A. Salaries. Give % time even if no salary is requested.
State names or "to be recruited"

	% Time	Amount
Professional Personnel including Principal Investigator		
Hans-Martin Jäck, Ph.D.	20%	0
Ulrike Keyna, Ph.D.	100%	0
Technical Support		
Gabriele Beck-Engeser	25%	7,573
Fringes @ 22.11%		1,674
	A. Salaries subtotal	9,247

B. Consumable supplies (by major category)

Fetal calf serum 1 liter per month @ $300 liter x 12 months	3,600
Restriction enzymes $500 per month x 12 months	6,000
Disposable plastics $450 per month x 12 months	5,400
PCR & sequencing kits	2,000
B. Consumables subtotal	17,000

C. Other Expenses (itemize)

Fluorescence cell sorter $1,000 month x 12 months	12,000
Oligonucleotides 20 x 30 bases @ $3.00 base	1,800
Mice: purchase 100 balbc month at $7.50 each x 12 months	9,000
per diem barrier @ $0.79 day/50 mice/365 days	14,417
per diem conven. @ $0.13 day/50 mice/365 days	2,373
Radioisotopes ^{32}PdCTP and ^{35}S-Methionine	2,640
C. Other Expenses subtotal	42,230
D. INDIRECT COSTS (15% OF A + B + C). D. Indirect Costs	10,272

E. Permanent Equipment (itemize)

None	E. Permanent Equipment subtotal 0
F.	TOTAL REQUEST 78,749

8. PROJECTED BUDGET AMOUNTS:

BUDGET PERIOD	Salaries, Supplies and Other expenses	Permanent Equipment *	Indirect Costs	TOTAL
Year 2	71,900	0	10,785	82,685
Year 3 if applicable	75,495	- 0* -	11,324	86,819

*You may not use CTR funds to purchase permanent equipment in the terminal grant year.

CURRENTLY ACTIVE GRANTS , CONTRACTS and OTHER SOURCES of FUNDS
List financial support (direct costs, only) from all sources, including own institution.

Title of Project	Sources (give grant numbers)	Total Value of Grant (direct costs)	Current Annual Amount Available to You	Date of Termination of Grant
Schweppe Career Development Award	Schweppe Foundation	45,000	15,000	6/30/95
The Role in B Cell Tumor Formation	NIH R29 AI33618	350,000	65,000	12/31/98
Junior Faculty Research Award	American Cancer Society JFRA-536	79,500	28,000 Faculty Salary Only	6/30/97

Identify and describe any overlap of this application with the above grants:

None

Indicate the total annual funds available to you this year for all research projects under your supervision.	$ 80,000

PENDING OR PLANNED

Title of Project	Sources (give grant numbers)	Total Value of Grant (direct costs)	Avg. Annual Amount Available to You	Total Duration (give inclusive dates)
Function of Surrogate Light Chains During Cell Maturation	American Cancer Society-Illinois Division	35,000	35,000	1/1/95-12/31/95

Identify and describe any overlap of this application with the above project.

If the Council for Tobacco Research grant is funded, all unused funds from the ACS grant will be returned.