Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6351–6355, October 1983
Immunology

Functional immunoglobulin M production after transfection of cloned immunoglobulin heavy and light chain genes into lymphoid cells

(protoplast fusion/G418 selection)

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Communicated by Niels Kaj Jerne, July 11, 1983

ABSTRACT **The rearranged immunoglobulin heavy (μ) and light (κ) chain genes cloned from the Sp6 hybridoma cell line producing immunoglobulin M specific for the hapten 2,4,6-trinitrophenyl were inserted into the transfer vector pSV2-neo and introduced into various plasmacytoma and hybridoma cell lines. The transfer of the μ and κ genes resulted in the production of pentameric, hapten-specific, functional IgM.**

Work over the last decades has provided extensive information on immunoglobulin function and structure (1). Despite this information, it has been possible only in gross terms to relate molecular function with particular structural features.

With the advent of genetic engineering and gene transfer techniques, questions regarding structure–function relationships can now be readily addressed—that is, virtually any gene segment can be modified precisely *in vitro* and the novel segment can then be exchanged with its normal counterpart. By introducing such engineered genes into the appropriate cells, the effects of systematic alterations in protein structure on protein function can be assessed.

Because immunoglobulin production is a specialized function of cells of the B-lymphocyte lineage, it is expected that the conditions for proper Ig gene expression will be provided only in appropriate immunocompetent cells. For example, to produce normal pentameric IgM(κ), a cell must transcribe, process, and translate RNA for the μ and κ chains and also provide J protein, enzymes for the proper polymerization and glycosylation of the Ig chains, as well as a suitable secretory apparatus. We have previously described a system for transferring a functional immunoglobulin κ light chain gene into IgM-producing hybridoma cells (2). Here we extend this work to show that the transfer of the μ and κ chain genes of a defined specificity into various plasmacytoma and hybridoma cell lines results in the production of functional pentameric, hapten-specific IgM(κ).

MATERIALS AND METHODS

Cell Lines. X63Ag8 was originally derived (3) from the plasmacytoma MOPC21 and synthesizes IgG1(κ) of unknown specificity. X63Ag8.653 was derived from X63Ag8 as a subclone that synthesizes neither the heavy (γ1) nor light (κ) chain (4). Similarly, Sp2/0Ag14 is an Ig nonproducing subclone of the Sp2 hybridoma (5). Sp6 is a hybridoma making IgM(κ) specific for the hapten 2,4,6-trinitrophenyl (TNP); originally this cell line produced the γ1 and κ chains of X63Ag8 as well as the (TNP specific) μ_{TNP} and κ_{TNP} chains (6). A subclone of Sp6 not making the γ1 chain was isolated, and the Sp602 and Sp603 cell lines were derived from this γ1 nonproducer. The mutant cell line igm-10, derived from Sp602 (7), lacks the gene encoding μ_{TNP} (8).

Gene Transfer. The construction of pSV2-neo plasmid vectors carrying the genes for μ_{TNP} or κ_{TNP} or both is described in the text. The vectors were transfected into the $r_k^- m_k^-$ *Escherichia coli* strain K803. To transfer the vector, bacteria bearing the appropriate plasmids were converted to protoplasts and fused to the indicated cell lines as described (2). The frequency of G418-resistant transformants per input cell was approximately 10^{-4} for X63Ag8 and Sp2/0Ag14, 10^{-5} for igm-10, and 10^{-6} for X63Ag8.653.

Analysis of Ig. As described previously (7), Ig was biosynthetically labeled, in the presence or absence of tunicamycin, immunoprecipitated, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with or without disulfide bond reduction. TNP binding IgM was assayed by TNP-dependent hemagglutination and by TNP-dependent enzyme-linked immunoadsorbent assay (ELISA) as described (2, 7). The hemolyses of protein A-coupled erythrocytes and TNP-coupled erythrocytes were used to assay total IgM- and TNP-specific complement activating IgM, respectively (7).

Analysis of RNA and DNA. Cytoplasmic RNA was isolated according to Schibler *et al.* (9) and subjected to RNA blot analysis as described by Thomas (10).

Procedures for DNA extraction (11), nitrocellulose blotting (12), and radiolabeling of probes (13) have been described (14, 15). Probes specific for genes encoding immunoglobulin constant and variable regions are detailed in the figure legends.

RESULTS

Description of Vectors and Expression Systems. The hybridoma cell line Sp6 secretes IgM(κ) specific for the hapten TNP. We have previously described the cloning of the TNP-specific κ gene, designated Tκ1 (16), and the construction of the recombinant, pR-Tκ1, where Tκ1 is inserted in the *Bam*HI site of the vector pSV2-neo (2, 17). The μ_{TNP} gene was cloned in λCh4A from *Eco*RI partially digested DNA of Sp6 cells, and this clone is designated Sp6-718. The 16-kilobase-pair (kbp) fragment carrying the variable and constant regions was obtained from Sp6-718 after partial digestion with *Eco*RI and was inserted at the *Eco*RI site of the vectors pSV2-neo and pR-Tκ1. In these recombinants, designated pR-Sp6 and pR-HL_{TNP}, re-

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Abbreviations: TNP, 2,4,6-trinitrophenyl; ELISA, enzyme-linked immunoadsorbent assay; kbp, kilobase pair(s); SV40, simian virus 40; kb, kilobase(s).

spectively, the μ_{TNP} gene lies in the same orientation as the κ_{TNP} gene in pR-Tκ1—i.e., the direction of transcription of μ_{TNP} is opposite that of the simian virus 40 (SV40) early promoter (Fig. 1).

The mutant cell lines igk-14 and igm-10 that lack the κ_{TNP} gene and μ_{TNP} gene, respectively, were originally isolated from subclones of Sp6 (7). We have previously used igk-14 as a recipient cell line to assay expression of the κ_{TNP} gene (2). Expression of the μ_{TNP} gene of pR-Sp6 was assayed here in igm-10. The simultaneous production of both μ_{TNP} and κ_{TNP} chains from the vector pR-HL_{TNP} is assayed in X63Ag8, the IgG1-producing plasmacytoma parent of the Sp6 hybridoma. In later experiments the pR-HL_{TNP} vector was assayed in the non-producing cell lines Sp2/0Ag14 and X63Ag8.653. IgM production by the transformants is compared with Sp603, a subclone of the Sp6 hybridoma.

Selection of IgM(κ)-Positive Transformants. The recombinant plasmid vectors bearing the Ig genes also contain the bacterial gene *neo*, which renders the recipient cells resistant to the antibiotic G418 (17). To transfer the Ig genes into the hybridoma and plasmacytoma cells, bacteria harboring the recombinant plasmids were converted to protoplasts and fused with the various cell lines and G418-resistant cells were selected. Depending on the cell line, the efficiency of G418-resistant colonies ranged between 10^{-4} and 10^{-6} per input hybridoma or plasmacytoma cell (see *Materials and Methods*). The culture supernatant of G418-resistant colonies was tested for TNP-specific IgM by using either a TNP-specific ELISA or by assaying agglutination of TNP-coupled erythrocytes. In various experiments between 15% and 75% of the colonies were positive in such tests.

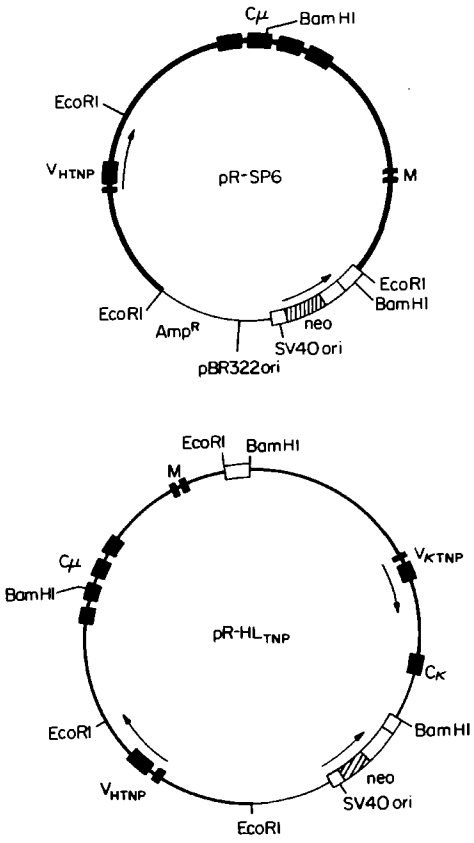

FIG. 1. Structure of the pR-Sp6 and pR-HL_{TNP} plasmids. pR-Sp6 contains the functionally rearranged μ_{TNP} gene (≈16 kbp), which was inserted into the *Eco*RI site of pSV2-neo (see text). In addition to the μ_{TNP} gene, pRHL_{TNP} contains the functionally rearranged κ_{TNP} gene (9.6 kbp) at the *Bam*HI site (2). Ig genes are represented by heavy dark lines. The directions of transcription of the Ig genes and the SV40 early region are indicated by arrows. The μ and κ exons are shown as filled boxes. M denotes alternative COOH-terminal coding regions that are utilized in the synthesis of membrane IgM. Thin lines are of pBR322 origin. The white boxes denote DNA derived from SV40, into which the bacterial gene conferring neomycin resistance (hatched box) has been inserted. For specific details concerning the pSV2-neo transfer vector (donated by P. Berg), see ref. 17.

Analysis of μ_{TNP} and κ_{TNP} Production. Colonies that were positive for TNP-specific IgM were cloned by limiting dilution and examined further. The transformant IR44L1, derived from the κ_{TNP}-positive cell line igm-10 and the μ_{TNP} vector pR-Sp6, makes about 25% of the normal (Sp603) amount of IgM, as measured by the TNP-dependent ELISA. The transformant XR19L4, derived from the cell line X63Ag8 and the μ_{TNP} + κ_{TNP} vector pR-HL_{TNP}, makes about 10% of the normal amount of IgM.

To examine the μ_{TNP} and κ_{TNP} separately, these chains were radiolabeled and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2). The Sp603 hybridoma cell line still makes the κ chain of its plasmacytoma parent, X63Ag8 (Fig. 2, lane a), as well as the specific μ_{TNP} and κ_{TNP} chains (Fig. 2, lane e). The XR19L4 transformant derived from X63Ag8 has two additional bands (Fig. 2, lane b), which comigrate with the μ_{TNP} and κ_{TNP} of Sp603. The igm-10 cells used here make κ_{TNP} but have ceased to produce the κ of X63Ag8 (Fig. 2, lane c), presumably because of a rearrangement in this κ gene (see legend to Fig. 5). The IR44L1 transformant derived from igm-10 has one new band that comigrates with μ_{TNP} (Fig. 2, lane d). As shown in Fig. 3, analysis of unreduced IgM by $NaDodSO_4$/polyacrylamide gel electrophoresis indicates that the transformants make predominantly pentameric IgM [$(\mu_2\kappa_2)_5$].

RNA Production. To examine the RNAs expressed by the transferred μ_{TNP} and κ_{TNP} genes, cytoplasmic RNA from the transformants was fractionated by gel electrophoresis and probed

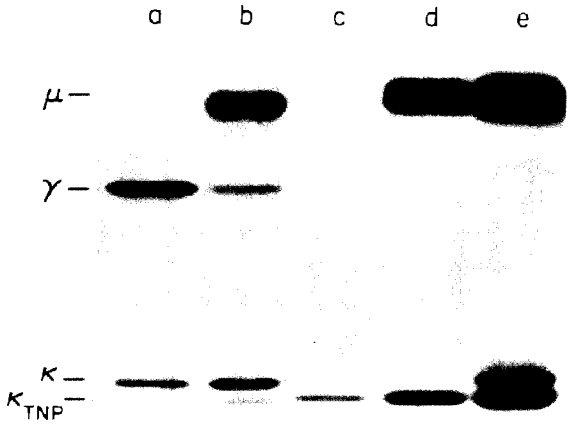

FIG. 2. Analysis of heavy and light chains of secreted Ig. G418-resistant transformant clones were biosynthetically radiolabeled with [^{14}C]leucine as described (7). Secreted immunoglobulins were immunoprecipitated with rabbit anti-mouse IgM antibody complexed with protein A-Sepharose CL-4B beads (Pharmacia). The precipitated material was reduced with 2-mercaptoethanol and analyzed by electrophoresis on a $NaDodSO_4$/polyacrylamide gel. Lane a, X63Ag8; lane b, XR19L4; lane c, igm-10; lane d, IR44L1; and lane e, wild-type hybridoma Sp603.

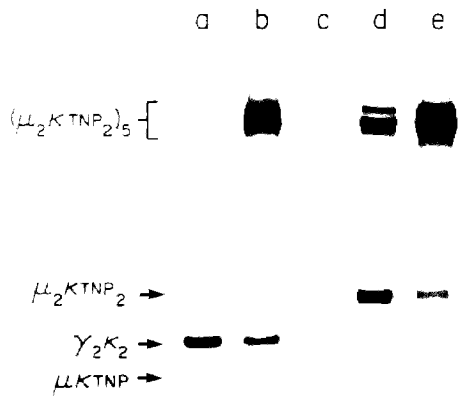

FIG. 3. Analysis of secreted (unreduced) Ig. The radiolabeled culture supernatants as described in the legend to Fig. 2 were analyzed by electrophoresis on a $NaDodSO_4$/polyacrylamide gel without reducing the disulfide bonds (7). Lane a, X63Ag8; lane b, XR19L4; lane c, igm-10; lane d, IR44L1; and lane e, wild-type hybridoma Sp603. The markers indicate the major forms of Sp603 IgM and X63Ag8 IgG1.

with various μ- and κ-specific DNA sequences (Fig. 4). RNA for the μ heavy chain was detected with a probe from the $C_\mu 4$ region. The transformants XR19L4 and IR44L1 have bands at both 2.7 and 2.4 kilobases (kb), whereas the parental hybridoma Sp603 has only one band at 2.4 kb (Fig. 4A). A genomic probe containing the μ membrane-specific exon hybridized only to the 2.7-kb band (data not shown). RNAs of 2.7 and 2.4 kb have been found to encode the membrane (μ_m) and secreted (μ_s) forms of the μ chain, respectively (19–21). These results suggest that, whereas Sp603 makes RNA only for the μ_s form, the transformants make RNAs for both μ_m and μ_s. However, we have been unable to detect membrane IgM by staining with fluorescent μ-specific antibodies. The μ_m form has a longer polypeptide chain than does the μ_s form and consequently can be distinguished from μ_s by its lower mobility in $NaDodSO_4$/polyacrylamide gel electrophoresis. Therefore, we examined intracellular μ chains that were biosynthetically radiolabeled in the presence of tunicamycin; for each transformant we found only one μ band, and this band comigrated with the μ band of Sp6 (results not shown). These observations suggest that either the 2.7-kb RNA is not translated or that the μ_m protein is very short-lived in the transformants.

In a similar manner, the RNA blots were hybridized with a probe derived from the κ_{TNP} V region. Compared to Sp603 and igm-10, the transformant XR19L4 was found to make a low amount of a 1.2-kb RNA that comigrated with authentic κ_{TNP} RNA (Fig. 4*B*).

Structure of Transferred DNA. To analyze the organization of the transferred pR-Sp6 and pR-HL_{TNP} plasmids in the transformed cell lines, *Bam*HI-digested cell DNA was hybridized with probes specific for the μ- and κ-chain constant region gene segments. The $C_\mu 1$–2 probe used here spans the *Bam*HI restriction site in the $C_\mu 2$ exon (Fig. 1). Therefore, a minimum of two fragments is expected to be detected with this probe.

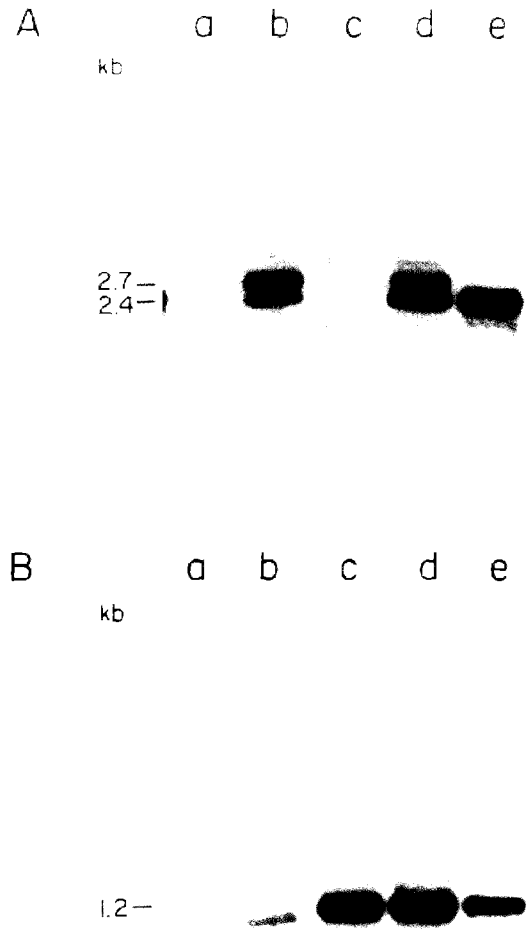

FIG. 4. Detection of μ_{TNP} and κ_{TNP} gene sequences in cytoplasmic RNA from transformed cell lines. Lanes a, X63Ag8; lanes b, XR19L4; lanes c, igm-10; lanes d, IR44L1; and lanes e, Sp603. Ten micrograms of total cytoplasmic RNA (9) was denatured with glyoxal, electrophoresed through a horizontal 1% agarose gel in 10 mM sodium phosphate buffer at pH 6.9, and transferred to nitrocellulose as described by Thomas (10). (*A*) The blot was hybridized with a ^{32}P-labeled probe corresponding to the $C_\mu 4$ exon. This probe was isolated from the cDNA clone pH76μ17 (donated by J. Adams) after digestion with *Pst* I (18). (*B*) A similar blot was hybridized with a ^{32}P-labeled probe containing κ_{TNP} V-region coding sequences (16). Sizes were estimated by comparison to mouse ribosomal 28S and 18S RNA (4.7 and 2.0 kb, respectively).

Two fragments of 6.0 and 16 kbp were detected in the DNA of both of the transformants. These correspond to the fragments generated by *Bam*HI digestion of the intact pR-Sp6 and pR-HL_{TNP} plasmids (Fig. 5). In addition, one (XR19L4) or two (IR44L1) extra fragments could be detected in the DNA from these cell lines. In parallel experiments, sequences indicative of unintegrated pR-Tκ1 plasmids have not been detected in the low molecular weight fraction of the Hirt supernatants (25) of similarly transformed igk-14 cells (results not shown). Taken together, these results suggest that the transferred genes are tandemly integrated into the chromosomal DNA of the recipient cells.

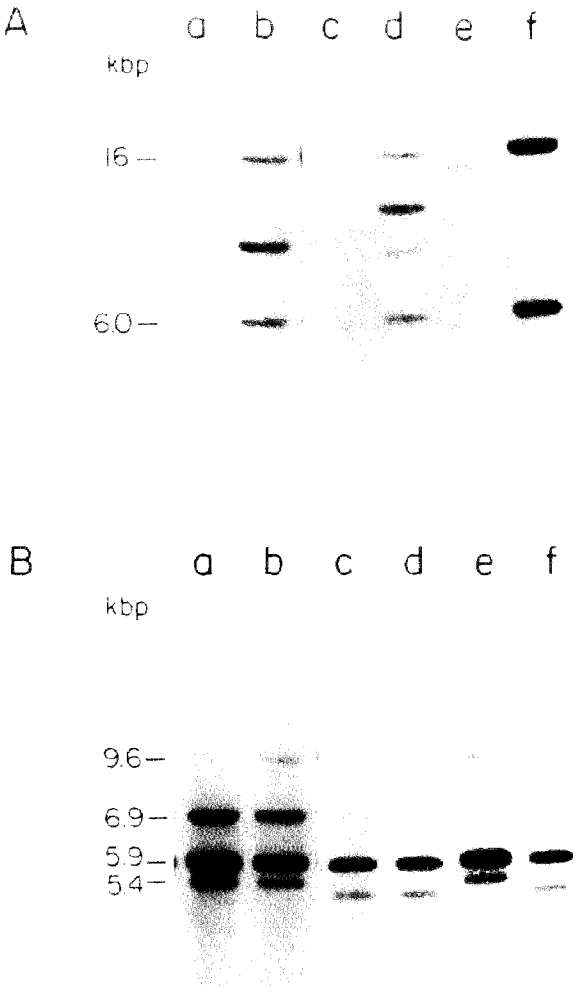

FIG. 5. Detection of pR-Sp6 and pR-HL$_{TNP}$ sequences in DNA from transformed cell lines. Lanes a, X63Ag8; lanes b, XR19L4; lanes c, igm-10; lanes d, IR44L1, lanes e, Sp603; and lanes f, igm-10 with 5 equivalents of pR-Sp6. *Bam*HI-digested DNA samples (20 μg) were electrophoresed through a 1% agarose gel at 2 V·cm^{-1} for 40 hr and transferred to nitrocellulose. (*A*) A previously hybridized blot (see *B*) was washed according to Thomas (10) and rehybridized to a ^{32}P-labeled probe containing the C$_\mu$1 and C$_\mu$2 exons. This probe was prepared by isolation of an appropriate fragment from a *Xba* I/*Hin*dIII digestion of a genomic clone of the μ-chain constant region gene segment. The bands corresponding to the μ-chain gene-containing fragments generated by *Bam*HI digestion of pR-Sp6 and pR-HL$_{TNP}$ are indicated. The two bands observed in lane e (11 and 14 kbp) correspond to the functionally rearranged μ_{TNP} gene in the wild-type Sp603 cell line. (*B*) The same blot was hybridized with a ^{32}P-labeled probe containing the κ-constant region gene segment that was isolated from the plasmid pL21-5 (donated by R. Wall) (22). The bands at 9.6 kb correspond to the κ_{TNP} gene (16). The bands at 6.9, 5.9, and 5.4 kbp correspond to rearranged κ chain genes present in the DNA of the X63Ag8 cell line (23, 24), two of which (5.9 and 5.4 kbp) were retained in the generation of the original Sp6 hybridoma. The 5.4-kbp band corresponds to the functionally rearranged X63Ag8 κ gene and this band is not observed in the case of igm-10 (lane c). Sizes were estimated by comparison to *Hin*dIII-digested λ phage DNA.

The pattern obtained for XR19L4 upon hybridization of the same blot with the C$_\kappa$ probe is consistent with the above interpretation. DNA from this transformant contains a 9.6-kbp fragment corresponding to the wild-type κ_{TNP} gene (16) in addition to other fragments that correspond to the κ chain genes endogenous to the recipient X63Ag8 cell line (23, 24).

Table 1. Assay of functional IgM

Cell line	Phenotype	Hemolysis titer on erythrocytes coupled with: Protein A	Hemolysis titer on erythrocytes coupled with: TNP	TNP/protein A ratio
Sp603	IgM, κ(TNP) + κ(X63)	2^4	2^6	4
igm-10	κ(TNP)	<1	<1	—
IR44L1		2^3	2^5	4
X63Ag8	IgG1, κ	<1	<1	—
XR19L4		2^3	<1	<1:8
Sp2/0Ag14	No Ig	<1	<1	—
SR1.2		2^4	2^6	4
SR40.1		2	2^2	2
X63Ag8.653	No Ig	<1	<1	—
X653R1.1		2^4	2^6	4

As described in the text, the transformants IR44L1 and XR19L4 were derived by introducing the μ_{TNP} gene alone or the μ_{TNP} and κ_{TNP} genes together into the igm-10 and X63Ag8 cell lines. Similarly, the cell lines SR1.2, SR40.1, and X653R1.1 were generated by transferring the μ_{TNP} + κ_{TNP} vector pR-HL$_{TNP}$ into Sp2/0Ag14 and X63Ag8.653. The indicated cell lines were grown to approximately 10^6 cells per ml, and culture supernatants were assayed for IgM concentration (lysis titer on protein A-coupled erythrocytes) and TNP-specific hemolysis activity (lysis titer on TNP-coupled erythrocytes). Culture supernatants were diluted serially 1:2 to obtain the end-point dilution (titer) that still caused lysis. The ratio of the TNP and the protein A titer is a measure of the specific activity of the secreted IgM.

Assay of IgM Function. We have tested the normal functioning of the IgM produced by the transformants by assaying its action in complement-dependent lysis of TNP-coupled erythrocytes (Table 1). The IgM concentration in the culture supernatants of the indicated cell lines was measured by the hemolysis of protein A-coupled erythrocytes in the presence of anti-IgM (7). These results indicate that IgM made by IR44L1 has normal activity with regard to TNP binding and complement activation. However, the transformant XR19L4 makes IgM that has an activity that is less than 1/30th of the normal activity in the TNP-dependent hemolysis assay. X63Ag8 still produces the myeloma κ chain, and this κ chain can be incorporated into IgM, thus reducing TNP-specific hemolysis activity (7). To avoid this problem of the nonspecific myeloma κ chain, the μ_{TNP} + κ_{TNP} vector pR-HL$_{TNP}$ was transferred into the nonproducer cell lines Sp2/0Ag14 (5) and X63Ag8.653 (4). The IgM produced by transformants of these cell lines has normal activity for TNP-specific hemolysis (Table 1).

DISCUSSION

We and others have previously reported the expression of Ig light chain genes in various cell types (2, 26–29). In this paper we have described the construction of plasmids that bear genes for TNP-specific immunoglobulin μ and κ chains. The expression of these genes was studied after the transfer of the plasmids into various cell lines derived from Ig-secreting plasmacytomas or hybridomas. The transfer of these plasmids into these cells is usually (see below) sufficient to cause the production of pentameric IgM(κ) that binds antigen (TNP) and activates complement—that is, these cell lines (X63Ag8, X63Ag8.653, igm-10, and Sp2/0Ag14) provide all of the machinery necessary for IgM production except the structural genes for the μ and κ

chains. The capacity to provide this machinery is present despite the fact that these cell lines have been propagated for years without overt selection for this property.

We expect that this system will be very useful in determining the structural requirements for normal IgM production and function. To date, the use of genetics for this purpose has been limited to the analysis of naturally occurring mutants that interfere with normal IgM processing and activity (7, 30). Although such mutants are useful as a starting point, *in vitro* mutagenesis offers a more rapid and systematic method of obtaining altered IgM. Thus, it should be possible to identify the amino acids that are critical for complement activation or Fc receptor binding. Similarly, one can expect to define the features that are necessary for pentamer formation, glycosylation, and secretion.

As is the case with other gene transfer systems, we have found that the various transformants produce quite different amounts of μ and κ chain, ranging from undetectable to approximately normal levels. In general, a linear relationship does not exist between the copy number of the transferred sequences and the level of Ig gene expression. Studies with transfer vectors presumed to be replication incompetent indicate that the transferred sequences integrate into different sites in the host chromosomes, independent of the method of transfer (31–33). Therefore, the context of the transferred genes is different from normal and different in each recipient. It is not known whether it is the different chromosomal locales that are responsible for the variation in the expression of the transferred genes or whether these results reflect a high frequency of mutation associated with the introduction of exogenous DNA into mammalian cells (34, 35).

The transformants XR19L4 and IR44L1 produce, in addition to a 2.4-kb RNA that comigrates with authentic μ_s RNA, a 2.7-kb RNA that appears to include the μ_m exon. As we have been unable to detect a μ_m protein, it is possible that the 2.7-kb RNA is aberrant in some respect (36–39). In contrast to the heavy chain gene results, the transferred κ chain genes in XR19L4 and in several transformants derived from igk-14 and the κ_{TNP} vector pR-Tκ1 (ref. 2; unpublished data) produce a single species of RNA that comigrates with authentic κ_{TNP} RNA.

We expect that the variations in the expression of the transferred genes will not interfere with the usefulness of this system in producing altered IgM for functional analysis. Furthermore, we anticipate that modifications of this protocol will allow investigation of the mechanisms controlling Ig gene expression.

Note Added in Proof. Gillies *et al.* (40) and Neuberger (41) have recently reported the expression of cloned heavy chain genes in transformed lymphoid cells.

We thank Nusrat Govindji and Catherine Filkin for expert technical assistance. This work was supported by grants from the Medical Research Council, the National Cancer Institute, the Arthritis Society, the Allstate Foundation, and Hoffmann-La Roche Ltd. A.O. was supported by a Terry Fox Cancer Research Fellowship from the National Cancer Institute. R.G.H. was supported by a studentship from the Medical Research Council.

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