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Deletion of the IgH enhancer does not reduce immunoglobulin heavy chain production of a hybridoma IgD class switch variant

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Immunoglobulin (Ig) gene promotors are active only in cells of the B-lymphocyte lineage. Transfection experiments have shown that this is due in part to tissue specific 'activating' DNA sequences, so called enhancers. It is not entirely clear whether these sequences are necessary for initial activation or also for maintenance of transcription of a gene. We describe here the isolation and characterisation of a mouse hybridoma cell line that has deleted *in vitro* the 'activating' sequence from the active IgH locus, the only IgH locus it contains. Nevertheless, Ig heavy chain production of the variant cell is not impaired and remains comparable with that of other hybridoma cells. Therefore, a high rate of Ig heavy chain production in antibody-producing cells is either independent of any sequences enhancing transcription or else these can easily be replaced by other DNA sequences with a similar function that have been moved into the vicinity of the V region.

Key words: murine hybridoma cell lines/isotype switch variants/fluorescence-activated cell sorting/IgH enhancer/immunoglobulin gene expression

Introduction

Enhancers or activators are short DNA sequences that act probably only *in cis* on gene promotors stimulating transcription of the respective genes. They were first discovered in viral genomes (Khoury and Gruss, 1983). An eukaryotic enhancer element has since been identified that ensures tissue-specific transcription of immunoglobulin heavy chain genes. This 'immunoglobulin heavy chain gene enhancer' is located within the J_H-$C\mu$ intron on a 0.8-kb *Xba*I-*Eco*RI restriction fragment on the 3′ side of J_H (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Neuberger, 1983).

In transfection experiments using plasmids with and without enhancer sequences, enhancers were necessary for a high level of transcription. However, these experiments did not reveal whether enhancers are needed only for tissue specific induction of transcription or whether they are also required for the maintenance of a high level of Ig production. In order to discriminate between these two possibilities one would have to analyse cells that have deleted the enhancing sequence from an already activated transcription unit and then compare transcription with and without enhancer. For Ig heavy chain (IgH) genes such an experimental system is provided by Ig class switch variants of mouse hybridoma cells. In most hybridomas class switch variants occur at frequencies of 10^{-5} to 10^{-7} per cell per generation (Sablitzky *et al.*, 1982). Usually the variant cells express the constant region (C_H) gene that is located 3′ next to the 'wild-type' C_H gene in the parental cells and the 'wild-type' C_H gene is deleted from the active IgH gene locus in the variant cells (Sablitzky *et al.*, 1982). We report here the isolation and characterisation of two hybridoma class switch variants that have switched from expression of $C\mu$ to that of $C\delta$. One of these variants has deleted in addition to the $C\mu$ gene the IgH enhancer.

Results and Discussion

Isotype variants in IgM producing hybridoma cell lines

The mouse hybridoma cell line 20.2–267 was obtained by fusion of a 4-hydroxy-nitro-phenylacetyl (NP) specific BALB/c splenic B cell with a X63.Ag8-653 cell and sub-cloned for stable expression of NP-specific IgM with λ light chains (White-Scharf *et al.*, 1982). We analysed clone 267.7 for class switch variants by staining cytoplasmic Ig of up to 10^6 cells with Ig class-specific fluorescent goat antibodies and found no variants expressing IgG. However, two out of 10^6 cells stained for IgD and not for IgM (Table I). We isolated IgD-producing (IgD^+) variants in two cycles of enrichment of surface IgD^+ cells by fluorescence-activated cell sorting and cloning of the sorted cells. Among the variant clones 267.7δ1 was chosen for further characterisation by serology and restriction analysis of IgH loci.

Another class switch variant was isolated from the mouse hybridoma cell line AN59. This line was obtained by fusion of a X63.Ag8-653-cell with a $(C57BL/6xBALB/c)F_1$ splenic B cell producing NP specific IgM. Like 267.7 the AN59 cell line was analysed for class switch variants. Again, we found only IgD^+ variants. Those occurred at a frequency of 370 cells per 10^6 (Table I). The variants were isolated by sorting IgD^+ cells twice and cloning the enriched population. Clone AN59δ1 was selected for characterisation. Neither in the IgM producing hybridoma cell lines 267.7 and AN59, nor in the IgD-producing variants 267.7δ2.8 and AN59δ2.3 could class switch variants be found expressing one of the IgG isotypes (Table I).

Restriction endonuclease analysis

From the restriction analysis of the IgH gene loci of parental and variant 267.7 and AN59 cells it is clear that the IgD producing class switch variants have deleted the $C\mu$ gene from the active IgH locus (Figures 1 and 2). AN59δ1 cells have in addition lost the entire inactive IgH locus of the 'b' haplotype as detected by J_H, $C\mu$ and $S\gamma1$ probes (data not shown). Both

Table I. Frequencies of isotype variants in 10^6 cells, determined by staining of cytoplasmic Ig with Ig class-specific fluorescent goat antibodies

Cell line	B1-8	B1-8δ1.8	267.7	267.7δ2.8	AN59	AN59δ2.3
Isotype						
μ	+	0	+	0	+	0
δ	1	+	2	+	370	+
γ3	0	0	0	0	0	0
γ1	0	0	0	0	0	0
γ2b	0	0	0	0	0	0
γ2a	0	0	0	0	0	0

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Fig. 1. Restriction analysis of the IgH loci of 267.7, 267.7δ1, AN59 and AN59δ1 cells. **(a)** Hybridization of the J_H3-J_H4 probe to *Bam*HI digested DNA. **(b)** Hybridization of the same filter to J_H probe. The filter was first hybridized to the J_H and then without washing to the J_H3-J_H4 probe. **(c)** Hybridization of the J_H3-J_H4 probe to *Xba*I digested DNA.

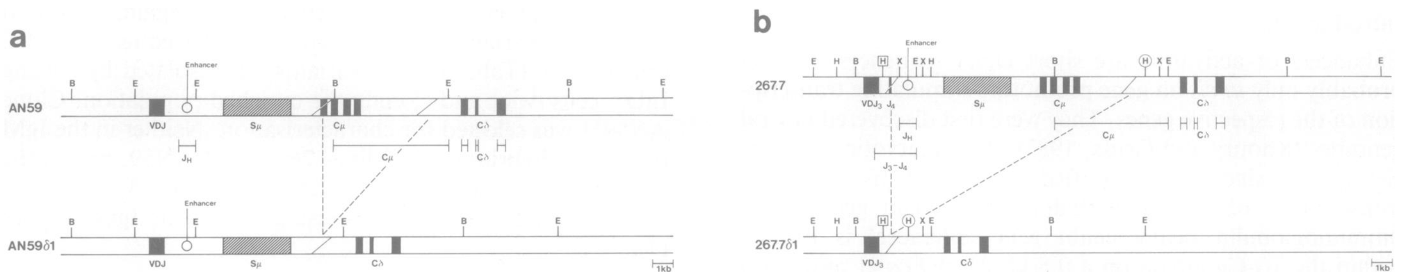

Fig. 2. Restriction maps of the IgH loci of 267.7, 267.7δ1, AN59 and AN59δ1. From the complete analysis we obtained the restriction map of the J_H-Cμ-Cδ regions of the active IgH loci of AN59 and AN59δ1 **(a)** and of 267.7 and 267.7δ1 **(b)**. Indicated are the restriction sites *Eco*RI (E), *Bam*HI (B), *Hind*III (H) or *Xba*I (X) that we have used, the intron (open), exon (closed), switch sequences (shaded), recombination sites (⁄) and probes used for analysis. The 267.7 and AN59 restriction maps are in accordance with the germline configuration downstream of J_H3 as determined earlier (Tucker *et al.*, 1980; Richards *et al.*, 1983; Liu *et al.*, 1980; Maki *et al.*, 1980; Sakano *et al.*, 1980). All cell lines except AN59 have only one IgH locus, thus allowing unequivalent co-ordination of restriction sites. AN59 has an inactive second IgH locus. Since this IgH locus is of the 'b' allotype the allotypic restriction site differences were used for assignment of restriction sites downstream of J_H to the active chromosome. The sites upstream of J_H could be co-ordinated since the variant AN59δ1 cells have lost the inactive IgH locus of AN59.

267.7 cell lines carry only a single IgH locus. The deletions removing the Cμ gene include VDJ-Cμ and Cμ-Cδ intron sequences to different extents in AN59δ1 and 267.7δ1. Regarding the 3′ end of the deletion, the *Eco*RI site 0.65 kb 5′ of Cδ in the germline and parental configuration is preserved in both variant cell lines. In AN59δ1 the recombination occurred 0.5 – 1.5 kb upstream of this site. In the 267.7δ1 cells a new 1.2 kb *Hind*III fragment is found with the J_H3-J_H4 probe and the 3′ *Hind*III site of this fragment is the one that is located 1.1 kb 5′ of Cδ. The 3′ end of the deletion in 267.7δ1 cells is thus within 600 bp upstream of this *Hind*III site. The 5′ end of the deletion in the AN59δ1 cells is located 0.1 – 1.1 kb upstream of the *Bam*HI site in the second Cμ exon. These cells still possesss the IgH enhancer and the complete Cμ switch sequence.

In 267.7δ1 cells the 5′ end of the deletion extends as close as 100 – 700 bp 3′ of the *Hind*III site between J_H3 and J_H4, removing also the *Xba*I site located 800 bp 3′ of this *Hind*III site in the germline configuration. A new 11.5 kb *Xba*-fragment hybridizing to the J_H3-J_H4 probe is found in 267.7δ1 since the next *Xba* site 3′ of J_H is now the one that is located 1 kb 5′ of Cδ in the parental cells (Figure 1c). The

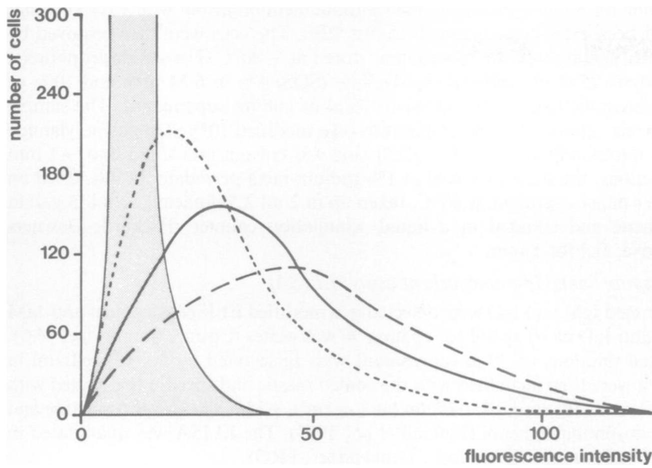

Fig. 3. Quantitation of cytoplasmic Ig in a fluorescence-activated cell sorter. Details for the preparation of the cells are given in Materials and methods. The fluorescence distribution of scatter positive 267.7 (shaded area), 267.7δ1(———), AN59δ1 (---) and B1-8δ1 (— —) cells is given as the number of cells exhibiting a given green fluorescence that increases linearly from channel 1 to 128. Each curve represents 10^4 cells.

Table II. Quantitation of Ig production

	Cytoplasmic[a]		Surface[b]		Secreted		
	IgD	IgM	IgD	IgM	Ig[c] (heavy chains)	IgM[d]	IgD[d]
B1-8	–[e]	–	–	17	100	10	–
B1-8.δ1	44	–	56	–	46	–	38.5
AN59	–	20	–	34	55	33.3	–
AN59δ1	20	–	35	–	21	–	6.6
267.7	5	31	6	38	34	100	–
267.7δ1	23	2	26	11	68	–	100

[a]Determined by flow cytometry as described in Materials and methods. Numbers for cytoplasmic IgD give the mean channel of the fluorescence distribution shown in Figure 2. Cytoplasmic IgM was determined analogous but the cells stained with a fluoresceinated goat anti-IgM instead of anti-IgD.
[b]Determined by flow cytometry as described in Materials and methods, except that the cells were not fixed and analysed alive. Numbers give the mean channel of the fluorescence distributions of cells stained with fluoresceinated goat anti-IgD or anti-IgM.
[c]Quantitated as described in Materials and methods. The radioactivity of the Ig heavy chains (as indicated in Figure 3) is given in percent relative to the one of the highest producer.
[d]Secreted IgM and IgD were detected in a modified ELISA. The relative concentration of IgM or IgD produced by a cell line is given as the relative titer relative to the highest titer.
[e]Not determined.

deletion of the IgH enhancer in 267.7δ1 cells is immediately evident since we could not detect any restriction fragment hybridizing to the J_H probe which is a 0.8 kb *Xba*I-*Eco*RI fragment that includes the 400 bp enhancer sequence (Figures 1b and 2b). Moreover, we have verified the restriction map of the 267.7δ1 IgH locus by analysing the cloned *Bam*HI fragment (S.Klein *et al.*, unpublished results).

Immunoglobulin gene expression

To analyse the effect of the enhancer deletion on Ig expression we compared the amount of Ig heavy chains produced by AN59, AN59δ1, 267.7, 267.7δ1, B1-8 and B1-8.δ1 cells. The B1-8.δ1 class switch variant had been isolated from the NP specific hybridoma B1-8 (Neuberger and Rajewsky, 1981) and like AN59δ1 has deleted the Cμ gene but not the Sμ and

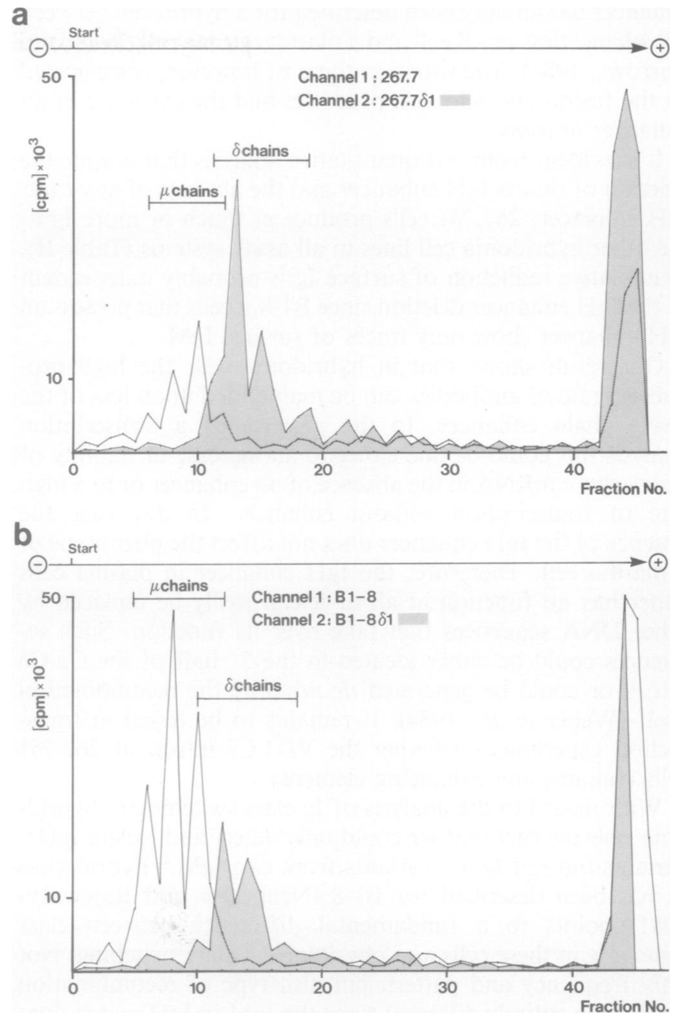

Fig. 4. Quantitation of secreted Ig. Details for the preparation are given in Materials and methods. The radioactive profile of an electrophoretic lane is given as counts per fraction. The profiles of (**a**) 267.7 and 267.7δ1 (shaded) and of (**b**) B1-8 and B1-8.δ1 (shaded) supernatants are each superimposed. The fractions containing Ig heavy chains are underlined. They are compared in Table II.

enhancer sequence (Sablitzky *et al.*, 1982).

The analysis of cells stained for cytoplasmic Ig by a fluorescence-activated cell sorter (Figure 3) allows the quantitation of cytoplasmic Ig on a per cell basis. Stained for cytoplasmic IgD the 267.7δ1 cells show an even distribution of fluorescence. There are no discrete high or low producing subpopulations. On the average 267.7δ1 cells contain more IgD than AN59δ1 and less than B-8.δ1 cells.

The amount of IgD secreted by 267.7δ1 cells is even higher than that secreted by B1-8.δ1 cells (Figure 4, Table II). This was quantitated by counting heavy and light Ig chains that had been biosynthetically labelled with [^{35}S]methionine and secreted by the cells into the culture supernatant. Since the δ constant region contains 7 methionines and an average V_H region ~1–3 methionines (Tucker *et al.*, 1980; Dildrop *et al.*, 1982), the V-region amino acid sequence differences between B1-8.δ1 and 267.7δ1 IgD are presumably negligible with regard to differential incorporation of [^{35}S]methionine.

The amount of secreted Ig was in addition quantitated by a solid phase radioimmunoassay (Table II). In accord with the biosynthetic labelling data, 267.7δ1 cells secrete more NP specific IgD than B1-8.δ1 and AN59δ1 cells.

A high rate of Ig production in the absence of a *cis*

enhancer has already been described for a hybrid cell between an enhancerless pre B cell and a plasmacytoma cell (Wabl and Burrows, 1984). The situation there is, however, complicated by the fusion and selection processes and the existence of an enhancer *in trans*.

It is evident from our quantitative analysis that despite the deletion of the *cis* IgH enhancer and the absence of any *trans* IgH enhancer, 267.7δ1 cells produce as much or more Ig as the other hybridoma cell lines in all assay systems (Table II). The relative reduction of surface Ig is probably independent of the IgH enhancer deletion since B1-8.μ cells that possess an IgH enhancer show only traces of surface IgM.

Our result shows that in hybridoma cells the high production rate of antibodies can be maintained upon loss of the heavy chain enhancer. In the absence of a transcription analysis this could be due either to an increase in stability of heavy chain mRNA in the absence of an enhancer or to a high rate of transcription without enhancer. In any case the absence of the IgH enhancer does not affect the phenotype of a plasma cell. Therefore, the IgH enhancer in plasma cells either has no function at all or it can easily be replaced by other DNA sequences that take over its function. Such sequences could be either located in the 3′ half of the Cμ-Cδ intron or could be generated *de novo* by the recombination itself (Weber *et al.*, 1984). It remains to be tested in transfection experiments whether the VDJ-Cδ intron of 267.7δ1 cells contains any enhancing elements.

With regard to the analysis of Ig class switching in hybridoma cells the fact that we could only detect and isolate IgD$^+$ variants but not IgG$^+$ variants from two IgM$^+$ hybridomas as has been described for B1-8 (Neuberger and Rajewsky, 1981) points to a fundamental difference between class switching in these cells and physiological class switching. Not only frequency and pattern but also type of recombination seem to be entirely different since the IgM to IgD switch does not involve recombination between switch regions.

Materials and methods

Cell culture

Hybridoma cells were grown in RPMI 1640, 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml).

Restriction endonuclease analysis

Genomic DNA was prepared, digested with the restriction endonucleases *Eco*RI (E), *Bam*HI (B), *Hind*III (H), or *Xba*I (X), size separated, blotted and hybridized to J_H (Alt *et al.*, 1981), J_H3-J_H4 (M.Börsch-Supan, personal communication), Cμ (Bothwell *et al.*, 1981), Cδ (Mushinski *et al.*, 1980) and Sγ1 (Shimizu *et al.*, 1982) specific probes (Sablitzky *et al.*, 1982). An aliquot of the cells used for DNA preparation was stained for cytoplasmic IgM or IgD. In all populations >90% of the cells were positive.

Immunofluorescence

For fluorescence microscopy and fluorescence activated cell sorting the cells were stained with Ig class specific fluorescent goat antibodies as previously described (Radbruch *et al.*, 1980; Liesegang *et al.*, 1978). $2-5 \times 10^6$ logarithmically growing cells of each population were fixed in ethanol/acetic acid (95:5, v:v) for 20 min at −20°C, washed twice with Dulbecco's phosphate buffered saline (DPBS) containing 5% FCS and stained with 30 μl of 0.1 mg/ml fluoresceinated goat anti-IgD for 30 min at room temperature. The goat anti-IgD was isolated from a goat anti-B1-8 IgM serum that had been absorbed extensively with B1-8 IgD coupled to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden), was eluted from a B1-8 IgD sorbent and tested in a solid phase radioimmunoassay (Klinman *et al.*, 1976). Fluorochrome-conjugation was carried out as previously described (Kearney *et al.*, 1976). The stained cells were washed twice and analysed in a modified FACS I (Becton-Dickinson, Mountain View, CA) (Weichel *et al.*, in preparation).

Quantitation of secreted Ig

1.5×10^6 logarithmically growing cells were grown with 250 μCi of [^{25}S]methionine for 5 h in 1 ml RPMI 1640 without methionine but with 10% FCS that had been extensively dialysed against PBS. The cells were then removed by centrifugation and the supernatant stored at −80°C. For gel electrophoretic analysis 25 μl of sodiumdodecylsulfate (SDS) 4% in 6 M urea and 10% of 2-mercaptoethanol were added to 25 μl of culture supernatant. The sample was size separated by electrophoresis on a modified 10% SDS-polyacrylamide gel (Holtkamp *et al.*, 1981). Each lane was cut out and sliced into ~1 mm fractions, the slices dissolved in 1% sodium-meta-perjodate for 4 h, dried on filter paper overnight at 80°C, taken up in 2 ml 2,5-diphenyloxazol (5 g/l) in toluene and counted in a liquid scintillation counter (Packard, Downers Grove, IL) for 1 min.

Enzyme linked immunosorbent assay (ELISA)

Secreted IgM and IgD were detected in a modified ELISA using goat anti-IgM or anti-IgD at 10 μg/ml for coating 96 well plates (Costar, Cambridge, MA). Serial dilutions of 30 μl supernatant with Ig secreted by 5×10^5 cells/ml in 72 h were then incubated with the coated plastic and specific Ig detected with goat anti-MIg coupled to biotin (at 2 μg/ml), avidin-alkaline phosphatase and p-phosphonitrophenol (Kendall *et al.*, 1983). The ELISA was quantitated in an ELISA reader (Dynatech, Denkendorf, FRG).

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