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Evidence for transient requirement of the IgH enhancer

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ABSTRACT

A transcriptional enhancer is thought to play a major role in determining tissue-specific expression of the immunoglobulin heavy chain (IgH) gene (1-3). However in three B-lymphoid cell lines the Ig enhancer has been lost due to a spontaneous deletion, yet heavy chain synthesis persists at a high level (4-6). In the case of the enhancerless delta chain gene (5) we wanted to test whether the IgH enhancer is no longer necessary for maintenance of transcription or whether perhaps a new enhancer was created *de novo* by the deletion process. To this end we have cloned the relevant portion of the variant IgH gene and transfected it into myeloma and hybridoma cells. We find that the reintroduced gene segment is not expressed unless it is linked again to an enhancer. The results suggest that the IgH enhancer is necessary for the onset of transcription, presumably by organizing the gene into stable transcription complexes, but is dispensable at later stages once the transcription unit is activated.

INTRODUCTION

During B-cell differentiation the genes which encode immunoglobulins undergo several rearrangements. The active heavy chain gene is formed by joining V_H, D and J_H sequences to form a complete V_HDJ_H variable region gene which is at first expressed together with the constant region Cμ (7-12). The resulting immunoglobulins belong to the IgM class. In further development the same V_HDJ_H can switch from the expression together with Cμ to the expression with other constant region (C_H) genes, in a process which is called class switching (9,13,14).

Using the fluorescence-activated cell sorter we had selected murine hybridoma cell lines which had switched *in vitro* from IgM to IgD production. These class switch variants had deleted the Cμ region and one of them, designated 267.7 δ, had lost the IgH enhancer in addition to Cμ but nevertheless maintained a high

level of IgD production (5).

We were facing the apparent paradox that an endogenous heavy chain gene lacking an enhancer was expressed at a high level, yet cloned heavy chain genes require an enhancer for efficient expression when introduced into B-lymphocyte cells by transfection (2,3). In addition, the location of the IgH enhancer within the constant region and the strict cell type specificity suggested a function in IgH gene expression. Therefore, we considered it unlikely that the enhancer effect is a mere transfection artefact, as has been suggested by others (4). We decided to test some possible explanations for the persisting expression of the IgH gene after deletion of the enhancer sequence. Gene amplification, the most frequent mechanism of mammalian cells to compensate for inefficient expression of a mutant gene, could be ruled out since the enhancerless heavy chain gene is present at about one copy per cell and the inactive IgH locus is deleted (5). This latter fact also excludes the possibility that an enhancer present in the nonfunctional IgH locus could somehow complement the enhancerless gene. Several other possibilities, however, had to be considered:

1) The high level of Ig production does not correspond to a high level of mRNA, i.e. transcription of the enhancerless Ig gene is inefficient but is compensated by an increase in translation efficiency.

2) The particular cell line 267.7 δ has acquired the property to express any enhancerless IgH gene at a high level, perhaps due to the presence of a transactivating protein like Adenovirus Ela (15-17).

3) A new enhancer-like sequence has been formed within the recombined Ig heavy chain gene upon juxtaposition of the deletion endpoints.

4) Only the combination of the cloned and reintroduced δ gene in its own host cell would result in efficient expression e.g. due to the presence of a mutant transcription factor which would erroneously recognize the IgH deletion region as an enhancer.

5) Another, perhaps remote cellular enhancer substitutes for the deleted heavy chain enhancer.

6) There is a requirement for an enhancer to organize the Ig heavy chain gene into an active transcription unit but thereafter the enhancer becomes dispensable.

MATERIALS AND METHODS

Cells

Hybridoma cells 267.7μ, 267.7δ (5) and myeloma cells X63-Ag8 (18), a gift from S.Y. Chung, were grown in Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS), penicillin (100U/ml) and streptomycin (100μg/ml).

Transfection

0.33 μg of plasmid DNA was transfected together with DEAE-Dextran as described (1) into X63-Ag8 and 267.7δ cells.

Immunofluorescence

Thirty-six hours after transfection cells were fixed with methanol/acetone (70:30). For nuclear T-antigen cells were stained with a hamster anti-T-antigen serum (3h, 37°) and a FITC conjugated rabbit anti-hamster serum (1h, 37°). The number of cells staining for T-antigen was determined by fluorescence microscopy. The total number of cells was counted for 0.4mm^2, averaging 300-600 cells. The number of T-antigen positive cells was counted in the same area. For cells transfected with p24ß (table 1) the total number of cells was extrapolated from the number of cells in 0.4mm^2 for a 64mm^2 area, which was then screened for T-antigen positive cells.

Cytoplasmic Ig heavy chains were detected by staining the cells with FITC or TRITC coupled goat antibodies specific for the mouse IgM or IgD (5,19). Stained and unstained living cells were counted by fluorescence microscopy.

In an 8x8mm area of the plastic plate the total number of cells was extrapolated from counting smaller areas of 0.47mm^2 or 0.072mm^2.

A minimum of 500 cells was counted. The number of T-antigen positive and cytoplasmic Ig positive cells was counted in the same small area when the number of positive cells was in the range of percent. Otherwise the whole 8x8mm area was screened for positive cells.

The quality of DNA was controlled on minigels. Transfection

efficiencies were controlled by cotransfection with the plasmid pDept (1) containing the SV 40 enhancer in combination with T-antigen. 8-20% of the cotransfected cells expressed T-antigen.

RNA analysis

Dilutions (as indicated in figure 1) of total cytoplasmic RNA of the cell lines 267.7μ and 267.7δ were hybridized to ^{32}P-GTP labelled RNA transcribed in vitro from vector SP6/267.7. B1-8 RNA was used as a negative control. The SP6 mapping procedure was done as described (20). In vector SP64 a 1.3 kb Bam HI (B) - HindIII (H) fragment was inserted containing the VDJ region (spotted box) of p267.7D (Fig. 2).

Plasmids

Plasmids used in transfection experiments (figure 2)

pB1-8M is a modification of pSV-Vu1 (3). A 14.2 kb Eco RI (E) - XhoI (Xho) fragment including VDJ and the Cμ gene was cloned into pBR 327 (wavy line). The region where the immunoglobulin enhancer is located is indicated Ⓔ (1-3).

Plasmid p267.7D contains a 9.5 kb Bam HI (B) fragment which includes about 500 bp upstream of the cap site, the VDJ region (spotted box), Cδ (hatched box), and about 3 kb of 3' untranslated sequence not including the polyadenylation side of the IgHδ gene of cell line 267.7δ. The region of recombination is a 1.1 kb HindIII (H) fragment (indicated).

Plasmid p267.7M is a chimeric plasmid constructed from p267.7D and pB1-8M. It contains the 3.4 kb Bam HI(B)-Eco RI(E) fragment of plasmid p267.7D which includes the 267.7D VDJ gene (spotted box) and the entire recombination region. This 3.4 kb fragment was ligated to the 10.2 kb Eco RI-Xho I fragment of B1-8M which contains the Cμ gene.

p267.7MEN is identical with plasmid p267.7M except that it contains 5' of the VDJ gene a 0.6 kb Eco RI(E)-Bam HI(B) immunoglobulin enhancer bearing sequence from plasmid πUC IgE$^+$ (21).

RESULTS

RNA analysis

First of all, we wanted to verify that the enhancer-deletion cell line 267.7δ produces not only high levels of Ig protein but also a correspondingly high level of Ig m-RNA (possibility 1). To

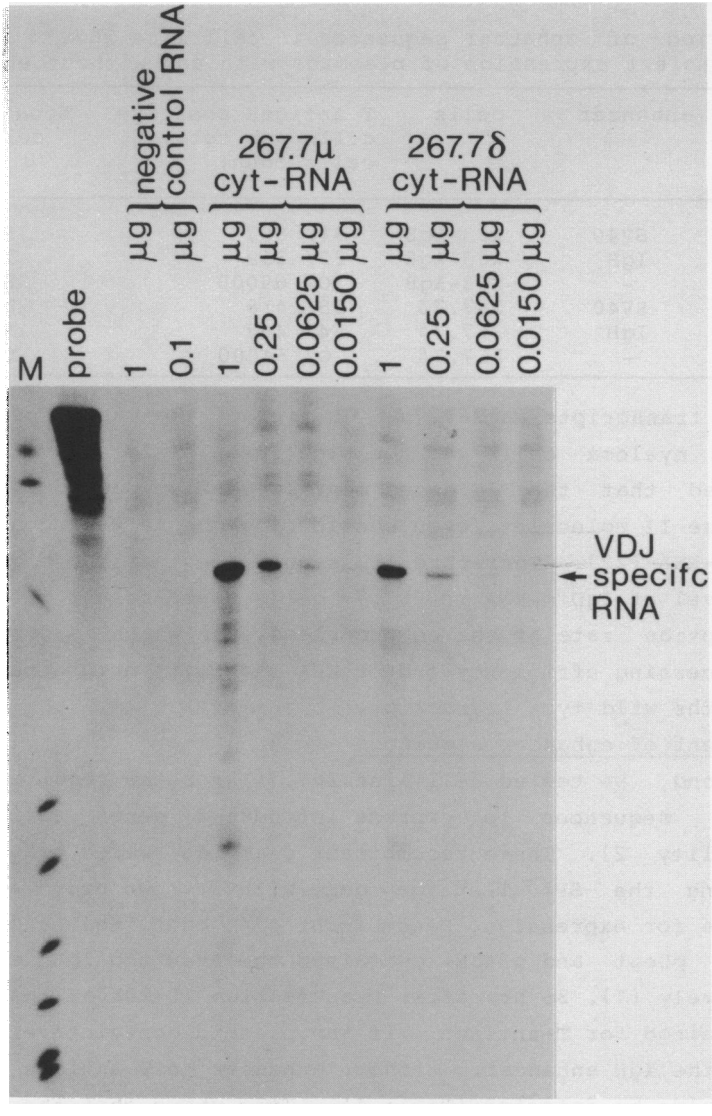

Figure 1:
Quantitation of Ig specific cytoplasmic RNA of the cell lines 267.7µ and 267.7δ. For further details see Materials and Methods.

this end, the amount of IgH transcripts produced by the enhancer-containing parental cell line 267.7µ and the enhancerless 267.7 δ class switch variant were compared. As shown in Fig. 1, the 267.7δ cell line contains at least 50% as much Ig heavy chain

Table 1:
Recognition of enhancer sequences in cell line 267.7δ and X63-Ag8. Transient expression of plasmids with and without enhancer.

plasmid	enhancer	cells	T antigen positive cells per total cells count	%positive cells
pDept	SV40	X63-Ag8	91/ 607	15
p24ßE+	IgH	X63-Ag8	27/ 359	7
p24ß	-	X63-Ag8	0/ 69000	0
pDept	SV40	267.7δ	85/ 418	20
p24ßE+	IgH	267.7δ	54/ 467	11
p24ß	-	267.7δ	0/ 54000	0

specific transcripts as 267.7µ. In view of the rapid proliferation of myeloma cells and the high level of Ig m-RNA it was calculated that the Ig genes must be densly packed with RNA polymerase II molecules, even considering the very long half live of Ig m-RNA (22). Therefore it is unlikely that the high steady state level of IgD m-RNA in 267.7δ cells could result from a low transcription rate of the enhancerless gene which is compensated by a processing efficiency and/or RNA stability much greater than that of the wild type transcript.

Requirement of enhancer sequences

Second, we tested cell line 267.7δ for the requirement of enhancer sequences to express introduced genes efficiently. (possibility 2). Three recombinant plasmids were tested, all containing the SV40 T-antigen gene with its own promoter as a test gene for expression. Recombinant p24ß contained no enhancer, whereas pDept and p24ßE+ contained the SV40 and IgH enhancer, respectively (1). 36 hrs after transfection 10-20% of the 267.7δ cells stained for T-antigen, if the plasmid contained either the SV40 or the IgH enhancer. Without enhancer no T-antigen expression could be detected (Table 1). This shows that the 267.7δ cells are able to recognize enhancer signals correctly and that a gene introduced into 267.7δ by transfection requires an enhancer for efficient transcription.

Searching for enhancer activities

It seemed, however, still possible that a new enhancer was created by the deletion process, in a manner similar to some rearranged SV40 deletion mutants (23,24). The endogenous heavy

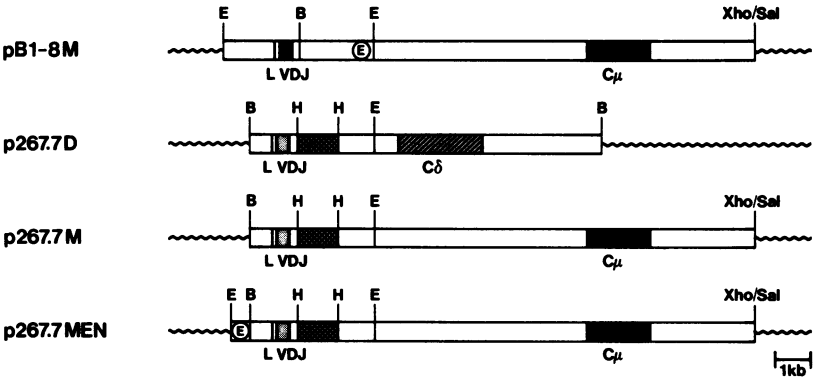

Figure 2:
Structure of plasmids used in transfection experiments (table 2)
Restriction sites: E=Eco RI, B=Bam HI, Xho=XhoI, Sal=SalI, H=Hind III.
DNA elements: L=leader, V=variable element, D=diversity element, J=joining element, Cμ=μ constant region gene, Cδ = δ constant region gene.
pBR327 sequences are indicated as wavy line. The region where the recombination has taken place is indicated between the two Hind III sites. The enhancer containing region is marked Ⓔ.

chain gene, after isolation and reintroduction into myeloma cells, would then be efficiently expressed even in the absence of the "classical" IgH enhancer (possibility 3). To test this, we prepared a genomic library of the 267.7δ cell line in phage L47.1 (25). From this library we isolated a 9.5 kb Bam HI fragment encompassing the VDJ and Cδ region of the expressed heavy chain gene. This fragment was subcloned into pBR 327 (p267,7D, Fig. 2).

In order to distinguish between expression of the reintroduced cloned gene and the endogenous one, p267.7D had to be modified. We constructed plasmid p267.7M (Fig. 2), where the critical deletion region including all upstream VDJ sequences was linked as a 3.4 kb Bam HI - Eco RI fragment to the Eco RI - Xho I Cμ region of plasmid pSV-Vμ1 (3).

As a control, recombinant pB1-8M (Fig. 2), which is a modification of pSV-Vμ1 (3), was also transfected. pB1-8M contains the VDJ gene segment of pSV-Vμ1 including the IgH enhancer with the Cμ constant region. As another control the IgH enhancer was placed upstream of the p267.7M gene to yield plasmid p267.7MEN (Fig. 2). Transfection into X63-Ag8 cells (an IgG1-secreting myeloma line) results in efficient expression of the chimeric

Table 2:
Transient expression of Ig heavy chains in cell lines X63-Ag8 and 267.7δ.

plasmid	cells	cIg positive cells per total cells counted	% positive for cIg
pB1-8M	X63-Ag8	21/1004	2
p267.7M	X63-Ag8	0/109000	0
p267.7MEN	X63-Ag8	36/1502	2
pB1-8M	267.7δ	11/513	2
p267.7M	267.7δ	0/20000	0
p267.7MEN	267.7δ	58/578	10

gene when the IgH enhancer is placed upstream of the modified enhancerless p267.7M gene (p267.7MEN), and, as expected with the pB1-8M control (Table 2). However, we could not detect expression from the enhancerless gene on recombinant p 267.7M. The used immunofluorescence assay would have allowed detection of the expression from the mutant IgH gene at levels as low as 5% as those observed with the wild-type constructs (not shown).

Testing the clone in its own producer cell

In order to address the fourth possibility, we had to introduce the enhancerless gene into its own cell line, 267.7δ, since it seemed possible that it could only be active in that particular cell line. The data (Table 2) show that the cloned enhancerless gene segment is not active unless it is linked to a new enhancer.

DISCUSSION

In our studies we have found that IgH gene transcription persists at a high level after spontaneous deletion of the IgH enhancer. The cloned gene segment harboring the deletion region is not expressed unless it is linked again to an enhancer when transfected into myeloma and hybridoma cells, thus excluding the possibility that a new enhancer was created by the deletion rearrangement. Recently the same conclusion was independently reached by Zaller and Eckhardt (26). These authors have cloned an IgG gene which was highly transcribed in the absence of an enhancer. Using stable transformation assays they have also found that the cloned gene was only active after linkage to an enhancer when

introduced into myeloma cells. Our results complement and extend those of Zaller and Eckhardt. We have reached our conclusions using different cells, a different gene, and a different transfection technique (transient expression versus stable transformation). In addition we obtained the same result when the enhancerless gene segment is transfected into its own producer cell, thus ruling out the obvious possibility that this cell line is mutated to efficiently express an enhancerless gene.

These experiments do not exclude the possibility 5 that in 267.7δ cells a remote cellular enhancer substitutes for the deleted Ig heavy chain enhancer. Given the ability of enhancers to act over distances of many thousand basepairs (27) this point is difficult to investigate since such a putative enhancer could well be outside of the VDJ-Cδ gene segment that we have cloned and analysed. So far, however, no additional IgH enhancer-like sequences have been found in the extended IgH region which has been tested for enhancer activity (1-3,28,29).

We consider the last possibility (6) particularly appealing, namely that the Ig enhancer is dispensable in B-cells. During B cell differentiation, induction of heavy chain gene transcription does not have to be reversible. Thus a high transcription rate of the IgH gene may persist even if the sequence which was originally responsible for the organization of the chromatin into stable transcription complexes is accidentally removed.

Based on studies of the transcriptional regulation of 5S RNA genes, Brown (30) proposed a model for how the activated transcription status of a gene could be propagated epigenetically throughout further cell proliferation. This model involves cooperative interaction of newly synthesized transcription factors with those that are still bound to either one of the sister chromatids in such a way that after mitosis complete active transcription complexes are regenerated in both daughter cells. Stable transcription complexes have also been described for ribosomal RNA genes (31) and for an U 2 RNA gene which is transcribed by RNA polymerase II (32).

Maintenance of active transcription complexes might also explain, as an indirect enhancer effect, the aberrant expression of c-myc when translocated to an Ig heavy chain locus by a recom-

bination event that removes the enhancer, a situation found in many myelomas and B lymphomas. This does not imply that enhancers generally are dispensable after the onset of transcription since the situation must be different for inducible genes such as metallothionein or interferon (33-36).

From the data presented in this paper and from previous results the following model of Ig heavy chain transcription can be proposed: At a "pro-pre-B cell stage", the heavy chain enhancer becomes active, presumably by binding celltype-specific factors, and the unrearranged heavy chain constant region starts to produce sterile transcripts (37,38). The variable regions are also transcribed, though only transiently (39). After V_H to DJ_H joining the active heavy chain gene is organized into a stable transcription complex. Finally, the presence of the IgH enhancer becomes dispensable. The IgH enhancer apparently is not required to maintain transcriptional activity after Ig class switching, i.e. introduction of new C_H-genes into the transcription unit, as we have shown for the 267.7 cell line.

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REFERENCES

1. Banerji,J., Olson,L. and Schaffner,W. (1983) Cell 33, 729-740.
2. Gillies,S.D., Morrison,S.L., Oi,V.T. and Tonegawa,S. (1983) Cell 33, 717-728.
3. Neuberger,M.S. (1983) EMBO J. 2, 1373-1378.
4. Wabl,M.R. and Burrows,P.D. (1984) Proc. Natl. Acad. Sci. USA 81, 2452-2455.

5. Klein,S., Sablitzky,F. and Radbruch,A. (1984) EMBO J. 3, 2473-2476.
6. Eckhard,L.A. and Birstein,B.K. (1985) Mol. Cell. Biol. 5, 856-868.
7. Early,P., Huang,H. Davies,M., Calame,K. and Hood,L. (1980) Cell 19, 981-992.
8. Sakano,H., Maki,R., Kurosawa,Y., Roeder,W. and Tonegawa,S. (1980) Nature 287, 676-683.
9. Nossal,G.J.V., Szenberg,A., Ada,G.L. and Austin,C.M. (1964) J. Exp. Med. 119, 485-502.
10. Melchers,F., von Boehmer,H. and Phillips,R.A. (1975) Transplant. Rev. 25, 26-58.
11. Cooper,M.D., Kearney,J.F., Lydyard,P.M., Grossi,C.E. and Lawton,A.R. (1976) Cold Spring Harbor Symp. Quant. Biol. 41, 139-145.
12. Raff,M.C. (1976) Cold Spring Harbor Symp. Quant. Biol. 41, 159-162.
13. Gearhart,P.J., Hurwitz,J.L. and Cebra,J.J. (1980) Proc. Natl. Acad. Sci. USA 77, 5424-5428.
14. Gearhart,P.J. and Cebra,J.J. (1981) J. Immunol. 127, 1030-1034.
15. Green,M.R., Treisman,R. and Maniatis,T. (1983) Cell 35, 137-148.
16. Treisman,R., Green,M.R. and Maniatis,T. (1983) Proc. Natl. Acad. Sci. USA 80, 7428-7432.
17. Svensson,C. and Akusjarvi,G. (1984) EMBO J. 3, 789-794.
18. Köhler,G. and Milstein,C. (1975) Nature 256, 495-497.
19. Radbruch,A. and Sablitzky,F. (1983) EMBO J. 2, 1929-1935.
20. Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis, T., Zinn,K. and Green,M.R. (1984) Nucleid Acids Res. 12, 7035-7056.
21. Gerster,T., Picard,D. and Schaffner,W. (1985) Cell, submitted
22. Schibler,U., Marcu,K.B. and Perry,R.P. (1978) Cell 15, 1495-1509.
23. Weber,F., De Villiers,J. and Schaffner,W. (1984) Cell 36, 983-992.
24. Swimmer,C. and Shenk,T. (1984) Proc. Natl. Acad. Sci. USA 81, 6652-6656.
25. Loenen,W.A. and Brammer,W.J. (1980) Gene 10, 249-256.
26. Zaller,D.M. and Eckhardt,L.A. (1985) Proc. Natl. Acad., in press
27. Banerji,J., Rusconi,S. and Schaffner,W. (1981) Cell 27, 299-308.
28. Mercola,M., Goverman,J., Mirell,C. and Calame,K. (1985) Science 227, 266-270.
29. Richards,J.E., Gilliam,A.C., Shen,A., Tucker,P.W. and Blattner,F.R. (1983) Nature 306, 483-487.
30. Brown,D.D. (1984) Cell 37, 359-365.
31. Reeder,R.H. (1984) Cell 38, 349-351.
32. Mattaj,I.W., Lenhard,S., Jiricny,J. and Robertis,E.M.De. (1985) Nature 316, 163-167.
33. Serfling,E., Lübbe,A., Dorsch-Häsler,K. and Schaffner,W. (1985) EMBO J. submitted.
34. Fujita,T., Ohno,S., Yasumitsu,H. and Tanigushi,T. (1985) Cell 41, 489-496.

35. Goodburn,S., Zinn,K. and Maniatis,T. (1985) Cell 41, 509-520.
36. Ryals,J., Dierks,P., Ragg,H. and Weissmann,C. (1985) Cell 41, 497-507.
37. Kemp,D.J., Harris,A.W., Cory,S. and Adams,J.M. (1980) Proc. Natl. Acad. Sci. USA 77, 2876-2880.
38. Alt,F.W., Rosenberg,N., Enea,V., Siden,E. and Baltimore,D. (1982) Mol. Cell. Biol. 2, 386-400.
39. Yancopoulos,G.D. and Alt,F.W. (1985) Cell 40, 271-281.