The EMBO Journal vol.9 no.3 pp.849-855, 1990

Extinction of Ig genes expression in myeloma × fibroblast somatic cell hybrids is accompanied by repression of the *oct-2* gene encoding a B-cell specific transcription factor

Yehudit Bergman[1], Batia Strich[1], Hava Sharir[1], Rosalie Ber[2] and Reuven Laskov[1]

[1]Hubert H.Humphrey Center for Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel and [2]Rappaport Family Institute for Research in the Medical Sciences, and the Faculty of Medicine, Technion, Haifa, Israel

Communicated by E.Klein

In most instances, fusion of differentiated cell types with fibroblasts has resulted in the extinction of differentiation-specific traits of the nonfibroblast parental cell. To explore the genetic basis of this phenomenon, we have used a series of somatic cell hybrids between myeloma cells and fibroblasts. Previous findings show that in these hybrids expression of the immunoglobulin (Ig) genes was extinguished at the transcriptional level. Our present results show that NF-κB transcription factor, known to be critical for κ-chain enhancer activity, is present although in a lower amount, in the nucleus and in the cytosolic fraction of most of these hybrids (probably attached to the previously postulated I-κB inhibitor). In contrast, the expression of the NF-A2/OTF-2 transcription factor encoded by the *oct-2* gene, which binds to the octameric motif located in the Ig promoters and heavy chain gene enhancer, is extinguished at the transcriptional level. Our data thus suggest that extinction of Ig genes expression occurs via an indirect mechanism in which a fibroblast factor suppresses transcription factor(s) which are critical for Ig transcription.

Key words: enhancer/negative regulation/promoter

Introduction

In order to understand the expression of tissue specific genes, it is necessary to understand not only the regulatory circuits that operate in expressing cells, but also the control mechanisms that play a role in inactivation of these genes in other cell types. One approach to the elucidation of the latter is that of somatic cell hybridization. A variety of such hybrids display either activation or suppression of tissue-specific products (Davis and Adelberg, 1973; Davidson, 1974). However, in most instances, fusion of differentiated cell types with fibroblasts has resulted in the extinction of differentiation-specific traits of the nonfibroblast parental cell (Davidson *et al.*, 1968; Schneider and Weiss, 1971; Sonnenschein *et al.*, 1971; Thompson and Gelehreter, 1971; Bertolotti and Weiss, 1972). Specific genetic loci responsible for the extinction of certain tissue specific genes were identified (Killary and Fournier, 1984; Petit *et al.*, 1986; Lem *et al.*, 1988).

One well-known example of extinction is the shut off of immunoglobulin (Ig) production in mouse myeloma × fibroblast cell hybrids (Periman, 1970; Coffino *et al.*, 1971). Several features make the κ-chain gene suitable for examination of the molecular mechanisms responsible for gene extinction in somatic cell hybrids. First, the regulatory elements involved in the κ-chain gene transcription are well defined (Queen and Baltimore, 1983; Bergman *et al.*, 1984; Picard and Schaffner, 1984, 1985; Queen and Stafford, 1984; Landolfi *et al.*, 1986; Sen and Baltimore, 1986a; Staudt *et al.*, 1986; Scheidereit *et al.*, 1987) and second, the actively rearranged κ genes can be easily identified and distinguished from the germ line counterparts. Detailed characterization of the κ-chain gene has identified at least two distinct *cis*-acting regions: the promoter and the enhancer. These elements independently direct tissue-specific transcription (Picard and Schaffner, 1984, 1985). The κ-chain gene promoter and enhancer consist of a modular array of sequence elements that are binding sites for *trans*-acting factors (Landolfi *et al.*, 1986; Sen and Baltimore, 1986a; Staudt *et al.*, 1986; Scheidereit *et al.*, 1987). Two *trans*-acting factors were found to dictate the specific expression of the κ-gene in B-cells: the NF-κB which binds to the B site in the κ-chain enhancer (Sen and Baltimore, 1986a) and the NF-A2/OTF-2A which binds to an octameric motif (ATTTGCAT) located within the upstream κ promoter and within the heavy chain promoter and enhancer (Landolfi *et al.*, 1986; Staudt *et al.*, 1986; Scheidereit *et al.*, 1987). Expression of NF-A2 transcription factor was found mainly in cells of the B lymphocyte lineage and constitutive expression of NF-κB is found in B cells and in macrophages (Griffin *et al.*, 1989). Recently a cDNA clone derived from the *oct-2* gene that specifies the NF-A2 binding protein was isolated and characterized (Muller *et al.*, 1988; Scheidereit *et al.*, 1988; Staudt *et al.*, 1988).

In our previous work we showed that myeloma × fibroblast hybrids do not produce Ig polypeptide chains and that extinction of Ig production occurs at the Ig transcriptional level (Greenberg *et al.*, 1987). More recent studies showed that heavy and κ light-chain gene promoters and the heavy chain enhancer are targets for down regulation in myeloma × fibroblast and myeloma × T-lymphoma cell hybrids (Junker *et al.*, 1988; Zaller *et al.*, 1988). Since NF-κB transcription factor is critical for κ-chain enhancer activity and NF-A2 transcription factor has been implicated as one of the major determinants for κ and heavy chain genes tissue specific expression (Landolfi *et al.*, 1986; Staudt *et al.*, 1986; Scheidereit *et al.*, 1987; Wirth *et al.*, 1987; Lenardo *et al.*, 1987), it was of interest to examine their presence in myeloma × fibroblast hybrids and to find out whether extinction takes place in an indirect manner through suppression of the activity of these transcription factors.

Our results show the presence of variable reduced levels of NF-κB binding activity in the nuclear extracts derived from individual myeloma × fibroblast hybrid clones. In contrast, the expression of the B-lymphoid specific transcription factor NF-A2 was extinguished at the transcriptional or early RNA processing levels in all the hybrid clones

examined. Our data thus suggest that extinction of both κ and heavy chain genes' expression in fibroblast × myeloma hybrids is due to the lack of NF-A2 transcription factor in these cell hybrids.

Results

To investigate the molecular mechanism of extinction of κ-gene expression, we used several independent myeloma × fibroblast hybrid cell lines. Such mouse−mouse hybrid cells have been shown to lose a few chromosomes in the first few generations following fusion. After this, the chromosomal making of the hybrids becomes stable (Ephrussi, 1972). All the hybrid cell lines examined in the present study adhered to the plastic, contained 5−20% fewer chromosomes than the sum number of their parental cells and retained the κ-chain gene of both parents. None of these hybrid cells contained a detectable level of κ-chain mRNA (Greenberg *et al.*, 1987, 1989).

NF-κB activity in myeloma × fibroblast hybrid cells

In order to see whether the extinction of Ig-gene expression correlates with the presence and activity of the κ-chain gene transcription factors we have used the electrophoretic DNA binding assay (Fried and Crothers, 1981; Garner and Revzin, 1981).

Previous analysis by the electrophoretic mobility shift assay has shown that the κ enhancer fragment binds at least three nuclear proteins in a sequence-specific manner (Sen and Baltimore, 1986a). One of these proteins, NF-κB, binds to the B site located in the 5′ *Dde*I−*Hae*III fragment of the κ enhancer (Sen and Baltimore, 1986a). Site specific mutations of the different sites has shown that the B site is critical for κ enhancer function and the other sites contribute to varying extent (Lenardo *et al.*, 1987). The NF-κB was originally identified as a B-cell specific protein, but subsequently was found to be present in an inactive form in the cytosol of non-B cell types. Its DNA binding activity was shown to be induced by a variety of treatments such as LPS, PMA, TNF-α, IL-1, etc. (Sen and Baltimore, 1986b; Lowenthal *et al.*, 1989; Shirakawa *et al.*, 1989). The other enhancer binding proteins are ubiquitously expressed in the nuclear extracts of both lymphoid and nonlymphoid cells (Sen and Baltimore, 1986a).

Since the activity of the κ-chain enhancer seems to be dependent on the presence of NF-κB protein in the nucleus, we have examined the presence of NF-κB in the nuclear extracts of the parental and hybrid cells. As expected, nuclear extracts from the 4T00.1 myeloma generated prominent retarded DNA−protein complexes (Figure 1, lane 8). Similarly to what was observed previously with extracts from T cells activated by PMA (Lowenthal *et al.*, 1989), nuclear extracts from myeloma cells yielded two retarded complexes with the NF-κB binding site; a faster complex and a more slowly migrating one. The intensity of the upper complex relative to the lower complex changed due to variations in salt concentration; in higher salt concentration the faster migrating complex was prominent (data not shown). These complexes are specific since they were competed off when the binding reactions were carried out in the presence of a 100 molar excess of the oligonucleotide encompassing the NF-κB binding site. In contrast, when 100 molar excess of the octa oligonucleotide was included, there was no

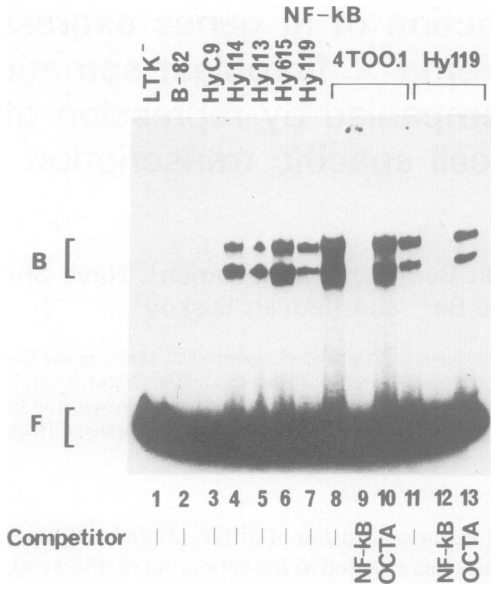

Fig. 1. Analysis of NF-κB activity in hybrid cells. Nuclear extracts were prepared from parental (LTK⁻, B82 and 4T00.1) and hybrid (HyC9, Hy113, Hy114, Hy119, Hy615) cells and assayed for the presence of NF-κB binding activity by gel mobility shift assay. End-labeled synthetic oligonucleotide encompasing the NF-κB site (10 000 c.p.m., 1 ng) was incubated with 15 μg of nuclear extract proteins in the presence of 2 μg of poly[d(IC)]. Binding reactions were done in the absence (**lanes 1−8, 11**) or in the presence of 100 molar excess of either a cold fragment containing an octamer oligonucleotide (**lanes 10, 13**) or a cold fragment containing the NF-κB oligonucleotide (**lanes 9, 12**). The source of each nuclear extract is shown above each lane and the position of nuclear factor bound and free DNA molecules are indicated by brackets marked B and F respectively.

significant competition (Figure 1, compare lane 8 to lanes 9 and 10). Extracts derived from the parental fibroblast cell lines LTK⁻ and B82 (Figure 1, lanes 1, 2) exhibit very low to undetectable levels of NF-κB binding activity, whereas in extracts derived from five independent hybrid cell lines (HyC9, Hy113, Hy114, Hy119, Hy615) the NF-κB activity ranged from a very low level (HyC9) similar to that found in the parental fibroblasts to relatively high levels (Hy615) which were ~2-fold lower than those found in myeloma nuclear extracts. We conclude that expression or activity of the NF-κB-like transcription factor is reduced to a variable extent but not extinguished in the myeloma × fibroblast hybrids relative to the myeloma parent.

NF-κB is detectable in the cytosolic fraction of hybrid cells after treatment with dissociating agents

It has been shown that the NF-κB transcription factor can be detected in two forms. The active form is found in nuclear extracts prepared from B cells and from phorbol ester (TPA) stimulated non-lymphoid cells. The 'non active' form is found in the cytosol of pre-B and non-lymphoid cells, and can be activated for binding in the presence of dissociating agents such as sodium deoxycholate (DOC). It has been suggested that in nonexpressing cells the NF-κB protein interacts with a cytosolic inhibitor which prevents its translocation to the nucleus and its DNA-binding activity (Baeuerle and Baltimore, 1988a,b). To determine whether our hybrid cells possess an inactive form of the NF-κB factor in the cytoplasm, nuclear extracts and cytosolic fractions

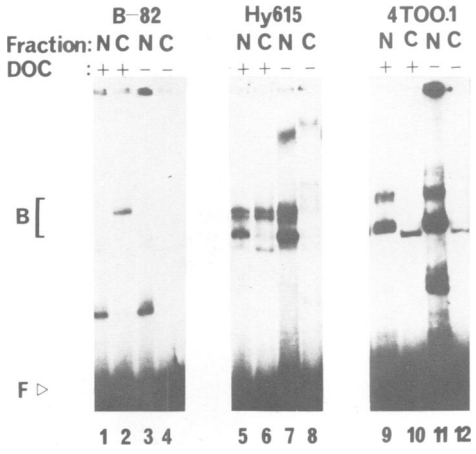

Fig. 2. The effect of DOC on the activity of NF-$\varkappa$B in subcellular fractions of parental and hybrid cells. Labeled NF-$\varkappa$B oligonucleotide was incubated with nuclear (N) and cytosolic (C) proteins derived from B82 fibroblast cells (**lanes 1–4**), hybrid 615 cells (**lanes 5–8**) and myeloma 4T00.1 cells (**lanes 9–12**) in the presence (+) or absence (−) of DOC. The DNA-binding reactions contained 4 μl of either nuclear or cytosolic extract in buffer (D^+), 2 μg of poly[d(IC)] in a final volume of 20 μl. The analysis of N and C proteins was done with the following amounts of proteins: B82, 10 μg; Hy615, 20 μg and 4T00.1, 15 μg. The position of nuclear factor bound (B) and free DNA molecules (F) are indicated. The 'B' marked retarded bands were specifically competed with cold NF-$\varkappa$B and not with cold octamer oligonucleotides. Hy615 nuclear proteins yielded another very slowly migrating complex which was also competed by cold NF-$\varkappa$B and not by cold octa oligonucleotide. This is probably a multivalent protein complex related to NF-$\varkappa$B protein but a definite answer should await a better biochemical characterization. The other bands observed are nonspecific since they were not competed off with cold NF-$\varkappa$B oligonucleotide.

from hybrid and parental cells were incubated with the NF-$\varkappa$B DNA binding site in the absence or presence of DOC prior to electrophoretic mobility shift assay. Without treatment, almost no NF-$\varkappa$B specific binding activity was detected in the cytosolic fractions of B82 and Hy615 (Figure 2, lanes 4 and 8). Upon treatment with 0.8% DOC and 1% NP-40, a dramatic increase in the amount of NF-$\varkappa$B binding activity was observed in the cytosolic fractions of both cell lines (Figure 2, lanes 2 and 6). Similar results were obtained with Hy113 (data not shown). Moreover these complexes were not found in binding reactions with a mutated NF-$\varkappa$B oligonucleotide sequence, which lacks the ability to bind to the NF-$\varkappa$B factor (Lenardo *et al.*, 1987), (data not shown). Compared to NF-$\varkappa$B found in the nucleus, relatively little NF-$\varkappa$B binding activity was observed in the myeloma cytosolic fractions and the level of NF-$\varkappa$B generated from the myeloma cytosolic and nuclear extracts was not significantly changed in the presence of DOC. The cytosolic and nuclear NF-$\varkappa$B were specifically competed off by cold NF-$\varkappa$B fragment and not by cold octa oligonucleotide (data not shown). It is concluded that similar to the parental fibroblast cells, the hybrids contain the cytosolic NF-$\varkappa$B that is probably attached to the presumptive cytosolic inhibitor (Baeuerle and Baltimore, 1988b).

NF-A2/OTF-2A activity is markedly repressed in myeloma × fibroblast hybrid cells

At least four proteins specifically recognize the octamer motif in nuclear extracts of mammalian cells. NF-A1/OTF-1 is a ubiquitous protein of apparent mol. wt 100 kd (Singh *et*

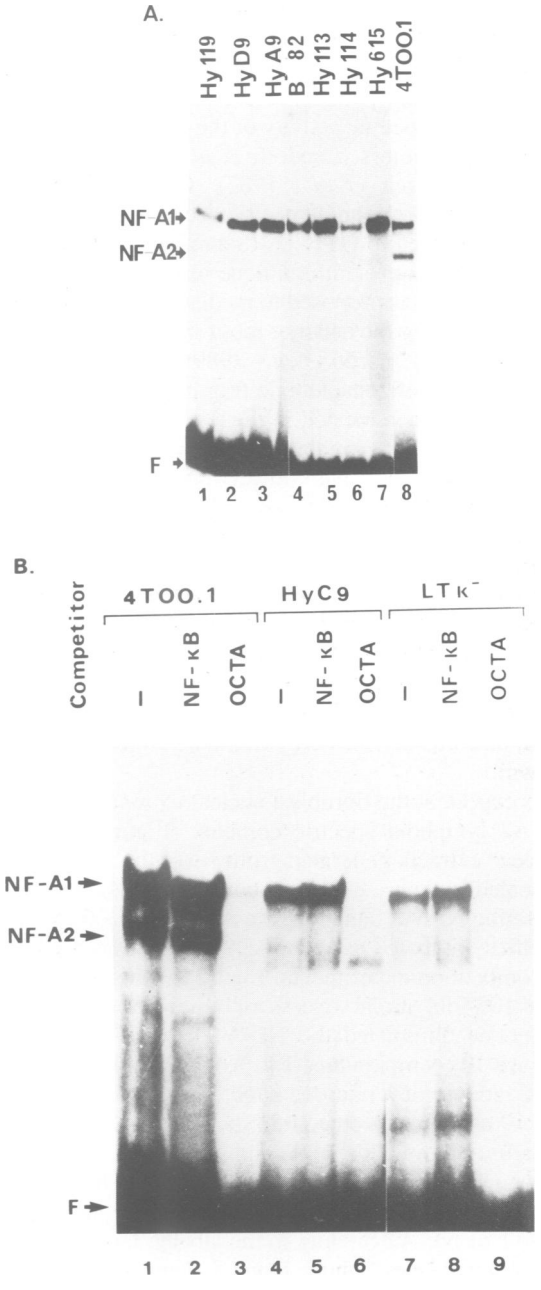

Fig. 3. NF-2A activity is repressed in myeloma × fibroblast hybrids. (**A**) Analysis of octamer binding proteins. Labeled octa synthetic nucleotide encompassing the NF-A2 binding site (100 000 c.p.m., 1 ng) was incubated with 15 μg of nuclear extracts prepared from parental (B82, 4T00.1) and hybrid (HyA9, HyD9, Hy113, Hy114, Hy119, Hy615) cells. Binding was done in the presence of 2 μg of poly[d(IC)] and analysis was performed by gel mobility shift assay. The source of each nuclear extract is shown either above or below each lane and the arrows denote the position of DNA molecules bound by NF-A1 or NF-A2. F indicates the position of free DNA.
(**B**) Specificity analysis. Octa labeled oligonucleotide was incubated wih 15 μg of nuclear extracts derived from myeloma 4T00.1 (**lanes 1–3**), hybrid C9 (**lanes 4–6**) and fibroblast LTK^- (**lanes 7–9**) in the presence of 2.0 μg of poly[d(IC)]. Binding reactions were done in the absence (**lanes 1, 4, 7**) or in the presence of 100 molar excess of either a cold fragment containing the NF-$\varkappa$B binding site (**lanes 2, 5, 8**) or a cold fragment containing the octamer oligonucleotide (**lanes 3, 6, 9**). The myeloma cells (4T00.1) contained a large quantity of the NF-A1 and NF-A2 bands that were specifically competed by the OCTA sequence. L-cells and the hybrids contained NF-A1. A very small amount of a retarded band which has the mobility of NF-A2 was detected in HyC9.

al., 1986; Sive and Roeder, 1986; Bohmann *et al.*, 1987; Sturm *et al.*, 1987). NF-A2 is restricted to lymphoid cells and has an apparent mol. wt of 60 kd (Scheidereit *et al.*, 1987). It was suggested that NF-A2/OTF-2A is responsible for the B-cell-specific activity of the octanucleotide element in the Ig promoters (Landolfi *et al.*, 1986; Staudt *et al.*, 1986; Scheidereit *et al.*, 1987). OTF-2B was recently described as a lymphoid-specific protein of ~75 kd which is closely related to OTF-2A (Schreiber *et al.*, 1988). The NF-A3 binding protein is unique to embryonal carcinoma cell lines and was proposed to mediate repression of certain genes containing the octamer motif in early stages of mouse development (Lenardo *et al.*, 1989).

We used an oligonucleotide fragment which contains the octamer sequence located in the $\varkappa$ promoter (Wirth *et al.*, 1987) as a target for binding of nuclear extracts from hybrids and parental cells. The slower-migrating protein–DNA complex observed in all lanes of Figure 3A is attributed to the binding of the ubiquitous NF-A1 octamer binding protein (Sturm *et al.*, 1987). The faster migrating band was prominent only in the nuclear extract from myeloma cells (Figure 3A, lane 8) and represents the lymphoid specific NF-A2 (Staudt *et al.*, 1986; Scheidereit *et al.*, 1987; Wirth *et al.*, 1987). On a longer exposure it was also possible to see the OTF-2B complex which migrated below the NF-A1 band, and was detected only in the myeloma cells (data not shown).

As expected, the fibroblast nuclear extract (B82) lacks the NF-A2 lymphoid specific complex (Figure 3A, lane 4). Nuclear extracts generated from seven hybrids tested (six presented in Figure 3A and one in Figure 3B) lack the faster migrating NF-A2 band (Figure 3A, lanes 1–3, 5–7). The specificity of these DNA–protein complexes is demonstrated by competition experiments. Figure 3B shows that addition of a 100-fold molar excess of unlabeled octamer oligonucleotide eliminated the NF-A1 and NF-A2 complexes (Figure 3B, compare lane 1 to 3, 4 to 6 and 7 to 9). A very small amount of a retarded band of a size similar to that of NF-A2 appeared in one out of the seven hybrids tested (C9 in Figure 3B) and this faint band was eliminated by octamer oligonucleotide (compare lanes 4 and 6). In contrast, an unlabeled NF-$\varkappa$B oligonucleotide did not interfere with NF-A1 or NF-A2 binding to the labeled target site (Figure 3B, compare lanes 1 and 2, 4 and 5, 7 and 8). The repression of NF-A2 binding activity in the hybrid extracts was not due to proteolytic degradation because NF-A1 showed similar complex formation in all nuclear extracts tested (Figure 3A and B). Thus, we can conclude that the mouse myeloma × fibroblast hybrids contain very little if any activity of the lymphoid specific NF-A2 transcription factors.

NF-A2/OTF-2A mRNA is absent in myeloma × fibroblast hybrids

Although the above results show that the NF-A2 is virtually absent from the hybrids' nuclear extracts, it is not clear whether the shut off is at the transcriptional level. We prepared pol(A)$^+$ mRNA from parental and from six of the hybrid cell lines which were analysed for NF-A2 binding activity. These mRNAs were analysed on a Northern blot using the recently isolated human oct-2 cDNA as a radioactive probe (Staudt *et al.*, 1988). It has been shown that the oct-2 cDNA encodes the NF-A2 transcription factor and it hybridizes to a number of mRNAs with lengths from

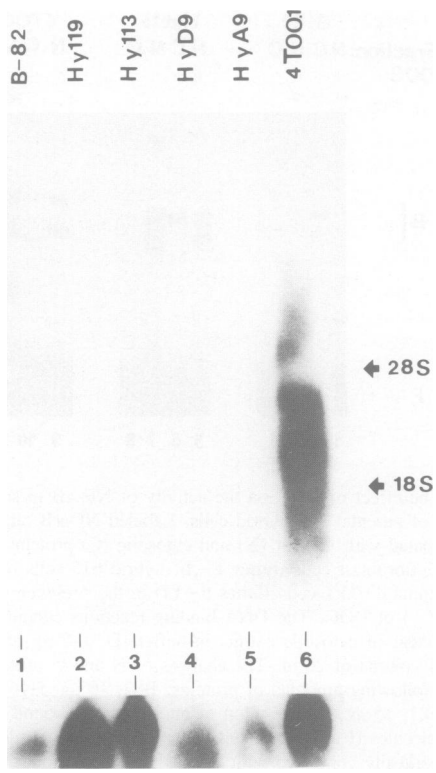

Fig. 4. Northern blot analysis of *oct-2* gene expression in parental and hybrid cell lines. Poly(A)$^+$ RNAs (10 μg) isolated from the parental (B82 and 4T00.1) and hybrid cells (Hy119, Hy113, HyD9, HyA9) were electrophoresed on 1.3% agarose–formaldehyde gels, transferred to nitrocellulose and hybridized with a 1.05 kb *Eco*RI DNA fragment containing the oct-2 cDNA sequence (Staudt *et al.*, 1988), washed under stringent conditions (0.1 SSC + 0.1% SDS at 68°C) and autoradiographed. The source of each RNA sample is shown above each lane. The blot was stripped and rehybridized with a β-actin cDNA probe. The blot containing the β-actin transcripts is shown in the lower part of this figure.

1 to 7 kb which are expressed only in B lymphoid cells (Clerc *et al.*, 1988; Muller *et al.*, 1988; Scheidereit *et al.*, 1988; Staudt *et al.*, 1988).

As expected, a complex pattern of hybridization of the oct-2 probe with RNA of myeloma cells was obtained, with different mRNA ranging in size from ~1 to 7 kb. Under high stringency washing conditions, no hybridization could be detected with mRNA extracted from the parental fibroblast and from six different hybrids (A9, D9, 113, 114, 119 and 615) including the four presented in Figure 4, lanes 2–5. The integrity of the mRNAs was monitored with a β-actin probe (Figure 3, lower part).

In order to see whether the *oct-2* gene is present in the hybrids, we have done a Southern blot analysis of the parental and hybrid cell DNA. Digestion with various restriction enzymes showed one to three main hybridization bands with the oct-2 probe (using stringent washing conditions). Digestion with *Hin*dIII revealed that all four hybrid DNAs tested (A9, 113, 114 and 615) contained three oct-2 hybridization bands of ~7.6, 3.9 and 1.7 kb in size. These bands were of an intensity quite similar to that of their parental cells, indicating that no preferential loss of *oct-2* genes has occurred (data not shown). Analysis with 10 different enzymes was performed in order to detect restriction fragment length polymorphism between the

parental myeloma cells (BALB/c) and L-cells (C3H) DNA, but none was found. Thus although we show that *oct-2* genes are present in our hybrids, we were unable to show that the alleles of the *oct-2* gene derived from the parental myeloma are present in the hybrids. However, we regard the possibility that the lack of NF-A2 DNA binding activity (and oct-2 mRNA) in the hybrids is simply due to chromosomal loss as unlikely. This is because a relatively small number of chromosomes (<20%) are lost in our hybrids (Greenberg *et al.*, 1987, 1989) and the chances that all the alleles (derived from the myeloma cells) coding for a certain transcription factor are concomitantly lost in all of the six independent hybrids analysed is very small, i.e. $<10^{-4}$ to $<10^{-8}$, assuming a random loss, and depending on whether there are one or two homologous chromosomes containing the *oct-2* gene per myeloma parental cell. Moreover, we have recently generated hybrids between myeloma and normal mouse fibroblasts which preserve the full set of their parental chromosomes and yet three of the hybrids that were examined do not express the *oct-2* gene (data not shown). Thus, our data indicate that the extinction of NF-A2 binding activity in the hybrid cells occurs at the level of mRNA transcription, either by regulation of transcription initiation, or by differential transcript stability.

Discussion

Extinction of differentiated properties in intertypic heterokaryons and hybrids is a well established phenomenon that has been shown to occur in many different hybrid systems.

In previous studies we have shown that in myeloma × fibroblast hybrids, the Ig expression is extinguished at the transcriptional level (Greenberg *et al.*, 1987). More recent studies provided evidence that the κ and heavy chain gene promoters and heavy chain gene enhancer are the targets for this repression (Junker *et al.*, 1988; Zaller *et al.*, 1988). It is reasonable to suggest that extinction of κ-chain gene can occur via two alternative mechanisms: (i) a direct one in which the fibroblast parent could contribute a negative factor(s) that binds directly to regulatory sequences within the κ-chain gene. Negative factors were postulated for the heavy chain enhancer (Kadesch *et al.*, 1986; Wasylyk and Wasylyk, 1986; Weinberger *et al.*, 1988) and for the κ-chain gene (Wall *et al.*, 1986). (ii) An indirect mechanism in which a fibroblast factor suppresses B-cell specific *trans* activators which are critical for κ-chain transcription and thus transcription is extinguished. An example of an indirect repression mechanism is the repression of heavy and κ-chain gene transcription by EIA products which do not bind directly to DNA (Hen *et al.*, 1985; Bergman and Shavit, 1988).

Extinction of κ-chain expression in our hybrids is unlikely to be due to structural changes in the κ-chain gene coding region for the following reasons: no aberrant rearrangements of the endogenous κ-chain gene have been detected and, furthermore, extinction also affects transfected (stable and transient) κ-chain genes (Junker *et al.*, 1988; Zaller *et al.*, 1988; our unpublished data).

Our results demonstrate that extinction of Ig expression in myeloma × fibroblast hybrids is probably mediated by an indirect mechanism, since the DNA binding activity of the B-cell specific transcription factor NF-A2 is either fully or almost fully extinguished. This seems to occur at the transcriptional or RNA processing level. In previously published studies this transcription factor was shown to be required for tissue specific activation of κ-chain promoter *in vivo* (Wirth *et al.*, 1987; Muller *et al.*, 1988) and *in vitro* (Scheidereit *et al.*, 1987). Therefore, the absence of this factor may account for the extinction of κ-chain transcription in the myeloma × fibroblast hybrid cells. Our results are compatible with the previously published data indicating that both κ and heavy chain genes are extinguished in myeloma × fibroblast hybrids (Greenberg *et al.*, 1987) and that the light and heavy chain promoter and heavy chain enhancer are the targets for this repression (Junker *et al.*, 1988; Zaller *et al.*, 1988), since these regulatory elements harbor the octameric motif to which the NF-A2 transcription factor binds. It is possible that in addition to an indirect effect on the activity of the Ig genes, the fibroblast cells also contain a dominant repressor that binds directly to one or more of the regulatory elements of the Ig genes. In fact, evidence for a direct suppressive mechanism through the octa sequence was recently found for the γ heavy chain gene in myeloma × T-lymphoma cell hybrids (Yu *et al.*, 1989).

Our results do not exclude the possibility that the NF-κB factor also plays a role in the extinction since its amount in the hybrid nuclei was lower than that in the myeloma parental cells and because of the existence of an inactive cytoplasmic form of NF-κB which resembles the NF-κB in the fibroblast cytosol (Baeuerle and Baltimore, 1988a,b).

Additional data supporting the contention that both direct and indirect mechanisms may take place in the extinction phenomenon are provided by studies carried out on growth hormone expression in rat pituitary cells × mouse fibroblast hybrids. In this system extinction was due to repression of expression of the cell type specific promoter transcription factor GHF-1 (McCormick *et al.*, 1988; Tripputi *et al.*, 1988) and probably also due to the activation of a direct repressor which binds to a silencer element that inhibits growth hormone promoter activity in nonpituitary cells (Tripputi *et al.*, 1988).

Regardless of the question whether a direct or indirect mechanism takes place in extinction, it may be mediated through a *de novo* methylation of CpG sites in the affected genes. Our preliminary results do not support this possibility since the *Hpa*II site in the variable region of the myeloma κ-chain gene does not undergo *de novo* methylation in two of our hybrids. The same site was found to undergo tissue specific demethylation during B-cell differentiation (Mather and Perry, 1983).

Studies using hepatoma × fibroblast hybrids suggest that fibroblast cells contain tissue specific extinguisher genes that are involved in the overall mechanism of tissue specific gene extinction (Killary and Fournier, 1984; Petit *et al.*, 1986; Lem *et al.*, 1988).

What is the biological significance of the occurrence of repressor(s) in fibroblast, which shut off the expression of other tissue specific genes? Since only a small fraction of the genome is expressed in each type of differentiated cell, specific repression mechanisms most probably play an important role in the commitment and decision processes, which determine cell type and lineage specificity. Thus, fibroblast repressor(s) may be part of a developmental program which determines the decision point between fibroblast and other cell lineages. The presumed repressor factor(s) may be responsible not only for the early fibroblast

commitment step, but also for the activation, and maintenance, of the fibroblast characteristic phenotype. Such a model explains the extinction of differentiated traits in many hybrid combinations between fibroblasts and other cell types, and predicts that extinction will affect many of the tissue specific genes in the non-fibroblast parental cells.

Materials and methods

Cells

All of the hybrid cell lines used in the present work were derived from two sets of fusion experiments using clones of the MPC 11 myeloma and L-cells: (i) 4T00.1L1 (a clone of MPC 11 cells producing $\varkappa$-chain only) × LTK^- cells, and (ii) 4T00.1 (producing , $\varkappa$) × B82 cells (a variant of LTK^- cells) (Greenberg *et al.*, 1987). The parental and hybrid cells were maintained in Dulbecco's medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Plasmids and DNA fragments

The plasmid p3.1 which contains the 1.05 kb *Eco*RI insert encompassing the human oct-2 cDNA fragment cloned into pUC18 vector was kindly provided by Dr David Baltimore (Staudt *et al.*, 1988). The plasmid β-actin which contains a 250 bp cDNA *Pst*I insert of the rat β-actin sequences was kindly provided by Dr Uri Nudel (Nudel *et al.*, 1982). The synthetic oligonucleotide encompassing the NF-$\varkappa$B site, 5'-GATCCAGAGGGG-ACTTTCCGAGAGGATC-3', was synthesized and cloned into the *Bam*HI site of pUC18 polylinker. The octa oligonucleotide 5'-GATCATGCA-AAT-3', was synthesized and cloned into the *Bam*HI site of the pUC18 polylinker. Both oligonucleotide containing plasmids were kindly obtained from Dr Ranjan Sen. The oligonucleotides were prepared by cutting at *Eco*RI and *Hin*dIII flanking sites within the polylinker. Fragments were isolated from 10% native polyacrylamide gels and quantitated by spotting onto ethidium bromide-containing plates (Maniatis *et al.*, 1982). As probes, these fragments were end-labeled by filling in the ends with the Klenow fragment of DNA polymerase I (Maniatis *et al.*, 1982).

Nuclear and cytosolic extracts

Nuclear extracts were prepared as described by Dignam *et al.* (1983). The cytosolic fraction was prepared as described by Baeuerle and Baltimore (1988a). Briefly, the postnuclear supernatant was centrifuged for 10 min at 4300 *g* and the resulting supernatant was ultracentrifuged for 1 h at 150 000 *g*. The supernatant (referred to as the cytosolic fraction) was adjusted to buffer D(+) by the addition of stock solutions.

DNA binding assays

The DNA binding reaction (20 μl) contained 10 mM Tris, pH 7.5, 20 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 2 μg poly[d(IC)], 10 000 c.p.m. (1 ng) of end-labeled DNA fragment and 15–30 μg protein of nuclear extract. For competition experiments, nonradioactive DNA fragments were included in the mixture in amounts detailed in the legends to the figures.

For analysis of cytosolic and nuclear fractions in the presence of DOC, the reaction mixtures were treated with 0.8% DOC followed by addition of DNA binding buffer containing 1% NP-40 as described (Baeuerle and Baltimore, 1988a). After incubation for 20 min at room temperature, samples were analysed by the electrophoresis mobility shift assay (Fried and Crothers, 1981; Garner and Revzin, 1981). The native 4% polyacrylamide gels (acrylamide:bisacrylamide ratio 30:1) containing 25 mM Tris–HCl (pH 8.5), 190 mM glycine and 1 mM EDTA buffer were electrophoresed for 3.5 h at 100 V. Gels were dried and autoradiographed with an intensifying screen at −70°C.

RNA blot analysis

Total cellular RNA was isolated by the guanidine thiocyanate method, and the poly(A)$^+$ mRNA was purified by oligo(dT) cellulose chromatography. The mRNA was subjected to electrophoresis through a formaldehyde-containing 1.3% agarose gel and transferred to a nitrocellulose or Nitran filter (Maniatis *et al.*, 1982). Hybridization was done as described using a random priming kit (Amersham) for labeling of the 1.05 kd cDNA segment containing the *oct-2* gene. The filters were washed in 0.2 × saline sodium citrate (SSC) and 0.1% SDS at 68°C and autoradiographed with an intensifying screen at −70°C. The filters were stripped by washing in 50% formamide, 10 mM Tris (pH 7.4) and 1 mM EDTA at 60°C for 1 h and rehybridized with ^{32}P-labeled β-actin cDNA probe (Nudel *et al.*, 1982) to control for the amount of mRNA loaded.

Acknowledgements

We thank Dr Uri Nudel for the β-actin probe, Dr Ranjan Sen for NF-$\varkappa$B and octa oligonucleotides and Dr David Baltimore for the oct-2 cDNA plasmid. We gratefully acknowledge the secretarial assistance of Ms Shoshana Skoll. This work was supported by grants from the Israel Academy of Sciences and Humanities and from the Paul Ehrlich Center for the study of normal and leukemic cells (to R.L. and Y.B.), and by a career development award from the Israel Cancer Research Fund (to Y.B.).

References

Baeuerle,P.A. and Baltimore,D. (1988a) *Cell*, **53**, 211–217.
Baeuerle,P.A. and Baltimore,D. (1988b) *Science*, **242**, 540–546.
Bergman,Y. and Shavit,D. (1988) *J. Immunol.*, **140**, 2076–2078.
Bergman,Y., Rice,D., Grosschedl,R. and Baltimore,D. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7041–7045.
Bertolotti,R. and Weiss,M.C. (1972) *Biochimie*, **54**, 195–201.
Bohmann,D., Keller,W., Dale,T., Scholer,H.R., Tebb,G. and Mattaj,I.A. (1987) *Nature*, **325**, 268–272.
Clerc,R.G., Corcoran,L.M., LeBowitz,J.H., Baltimore,D. and Sharp,P.A. (1988) *Genes Dev.*, **2**, 1570–1581.
Coffino,P., Knoweles,B., Nathenson,S.G. and Scharff,M.D. (1971) *Nature*, **231**, 87–90.
Davidson,R.L. (1974) *Annu. Rev. Genet.*, **8**, 195–218.
Davidson,R.L., Ephrussi,B. and Yamamoto,K. (1968) *J. Cell. Physiol.*, **72**, 115–128.
Davis,F.M. and Adelberg,E.A. (1973) *Bacteriol. Rev.*, **37**, 197–214.
Dignam,J.D., Lebowitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
Ephrussi,B. (1972) *Hybridization of Somatic Cells*. Princeton University Press, Princeton, NJ.
Fried,M. and Crothers,D. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
Garner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **9**, 3047–3060.
Greenberg,A., Ber,R., Kra-Oz,Z. and Laskov,R. (1987) *Mol. Cell. Biol.*, **7**, 936–939.
Greenberg,A., Hijazzi,M., Sharir,H., Cohen,L., Bergman,Y., Ber,R. and Laskov,R. (1989) *Int. J. Cancer*, **43**, 87–92.
Griffin,G.E., Leung,K., Folks,T.M., Kunkel,S. and Nabel,G.J. (1989) *Nature*, **339**, 70–73.
Hen,R., Borrelli,E. and Chambon,P. (1985) *Science*, **230**, 1391–1394.
Junker,S., Nielsen,V., Matthias,P. and Picard,D. (1988) *EMBO J.*, **7**, 3093–3098.
Kadesch,T., Zervos,D. and Ruezinky,D. (1986) *Nucleic Acids Res.*, **14**, 8209–8221.
Killary,A.M. and Fournier,R.E.K. (1984) *Cell*, **38**, 523–534.
Landolfi,N.F., Capra,J.D. and Tucker,P.W. (1986) *Nature*, **323**, 548–551.
Lem,J., Chin,A.C., Thayer,M.J., Leach,R.J. and Fournier,R.E.K. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7302–7306.
Lenardo,M.J., Pierce,J.W. and Baltimore,D. (1987) *Science*, **236**, 1573–1577.
Lenardo,M.J., Staudt,L.M., Robbins,P., Kuang,A., Mulligan,R.C. and Baltimore,D. (1989) *Science*, **243**, 544–546.
Lowenthal,J.W., Ballard,D.W., Bohnlein,E. and Green,W.C. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2331–2335.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mather,E.L. and Perry,R.P. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4689–4693.
McCormick,A., Wu,D., Castrillo,J.-L., Dana,S., Jennine,S., Thompson, E.B. and Karin,M. (1988) *Cell*, **55**, 379–389.
Muller,M.M., Ruppert,S., Schaffner,W. and Matthias,P. (1988) *Nature*, **336**, 544–551.
Nudel,U., Katcoff,D., Zakut,R., Shani,M., Carmon,Y., Finer,M., Czosnek,H., Ginsburg,I. and Yaffe,D. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2763–2767.
Periman,P. (1980) *Nature*, **228**, 1086–1087.
Petit,C., Levilliers,J., Ott,M. and Weiss,M.C. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2561–2565.
Picard,D. and Schaffner,W. (1984) *Nature*, **307**, 80–82.
Picard,D. and Schaffner,W. (1985) *EMBO J.*, **4**, 2831–2838.
Queen,C. and Baltimore,D. (1983) *Cell*, **33**, 741–748.
Queen,C. and Stafford,J. (1984) *Mol. Cell. Biol.*, **4**, 1042–1049.
Scheidereit,C., Heguy,A. and Roeder,R.G. (1987) *Cell*, **51**, 783–793.
Scheidereit,C., Cromlish,J.A, Gerster,T., Kawakami,K., Balmaceda,C.-G., Currie,R.A. and Roeder,R.G. (1988) *Nature*, **336**, 551–557.

Schneider,J.A. and Weiss,M.C. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 127–131.
Schreiber,E., Matthias,P., Muller,M.M. and Schaffner,W. (1988) *EMBO J.*, **7**, 4221–4229.
Sen,R. and Baltimore,D. (1986a) *Cell*, **46**, 705–716.
Sen,R. and Baltimore,D. (1986b) *Cell*, **47**, 921–928.
Shirakawa,F., Chedid,M., Suttles,J., Pollok,B.A. and Mizel,S.B. (1989) *Mol. Cell. Biol.*, **9**, 959–964.
Singh,H., Sen,R., Baltimore,D. and Sharp,P.A. (1986) *Nature*, **319**, 154–158.
Sive,H.L. and Roeder,R.G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6382–6386.
Sonnenschein,C., Richardson,I. and Tashjian,A.H., Jr (1971) *Exp. Cell Res.*, **69**, 336–344.
Staudt,L.M., Singh,H., Sen,R., Wirth,T., Sharp,P.A. and Baltimore,D. (1986) *Nature*, **323**, 640–643.
Staudt,L.M., Clerc,R.G., Singh,H., LeBowitz,J.H., Sharp,P.A. and Baltimore,D. (1988) *Science*, **241**, 577–580.
Sturm,R., Baumruker,T., Franza,B.R., Jr and Herr,W. (1987) *Genes Dev.*, **1**, 1177–1160.
Thompson,E.B. and Gelehreter,T.D. (1971) *Proc. Natl. Acad. Sci. USA*, **68**, 2589–2593.
Tripputi,P., Guerin,S.L. and Moore,D.D. (1988) *Science*, **241**, 1205–1207.
Wall,R., Briskin,M., Carter,C., Govan,H., Taylor,A. and Kincade,P. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 295–298.
Wasylyk,C. and Wasylyk,B. (1986) *Cell*, **45**, 553–560.
Weinberger,J., Jat,P.S. and Sharp,P.A. (1988) *Mol. Cell. Biol.*, **8**, 988–992.
Wirth,T., Staudt,L.M. and Baltimore,D. (1987) *Nature*, **329**, 174–178.
Yu,H., Porton,B., Shen,L. and Eckhardt,L.A. (1989) *Cell*, **58**, 441–448.
Zaller,D.M., Yu,H. and Eckhardt,L.A. (1988) *Mol. Cell. Biol.*, **8**, 1932–1939.

Received on September 4, 1989; revised on November 24, 1989