AN ENHANCER FACTOR DEFECT IN A MUTANT BURKITT LYMPHOMA CELL LINE

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RJ 2.2.5 is an immunoselected mutant of the Burkitt lymphoma line, Raji (1). It fails to display MHC class II antigens at the cell surface because of a lack of HLA-DR, -DP, and -DQ mRNAs (2, 3). The deficiency can be corrected by fusing RJ 2.2.5 cells with mouse cells expressing class II genes (3–5), or by transfecting them with mouse genomic DNA (6). Hence, RJ 2.2.5 has a defect in some positive regulatory factor capable of operating across species barriers. We have attempted to identify the target of this factor by asking whether a cell type–specific class II gene enhancer can function in RJ 2.2.5. In addition, we have compared DNA-binding proteins from Raji and RJ 2.2.5, concentrating on those that bind specifically to important sequence elements of the class II gene enhancer.

Materials and Methods

Cells. HA2 (short for A2-2.4c16) derives from a fusion between RJ 2.2.5 cells and mouse splenocytes (4) and carries only two mouse chromosomes, 16 and 17 (5). T-XI (short for T-XIS5.7) bears mouse genomic DNA stably integrated into the genome. It is a secondary transfectant, isolated as follows: mouse DNA was introduced into RJ 2.2.5 cells and the class II antigen–positive line T-V S4 was selected; DNA from T-V S4 was introduced into RJ 2.2.5 cells and the class II antigen–positive T-XI was selected (see reference 6).

Enhancer Test Constructs. Plasmids used to assay enhancer activity are diagrammed in Fig. 1. As illustrated in the lower left-hand corner, they all contain a rabbit β-globin reporter gene and an SV40 enhancerless promoter. Test fragments are cloned just 5′ to the SV40 promoter.

Transfection. 10 μg of plasmid DNA were transfected into 10^7 cells by the DEAE-Dextran technique (7). Enhancer activity was evaluated by quantitating, 2 d after transfection, the amount of RNA initiated at the SV40 early startsites. For each plasmid, at least three independent experiments were conducted, using two different DNA preparations.

S1 Nuclease Mapping. Isolation of RNA, RNA:DNA hybridization, S1 nuclease trimming, and analysis of the protected products on a denaturing polyacrylamide gel were performed as previously described (8). The single-stranded probe spans positions +137 to −352 on pAO (relative to the major transcriptional startsite), and was synthesized by the extension of a 5′ end-labeled oligonucleotide on single-stranded M13mp8βSV, as described (9).

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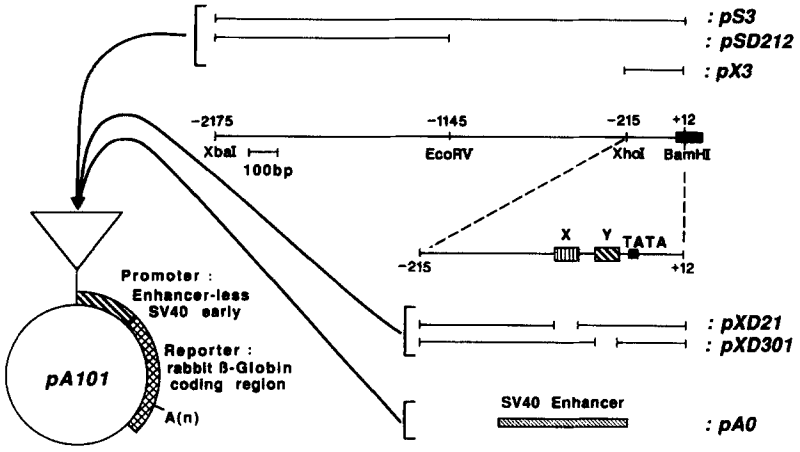


FIGURE 1. Constructs used to test enhancer function. The core common to all plasmids is pA101, diagrammed at the left. pA101 consists of a reporter, the rabbit β-globin coding region, and an enhancerless promoter, the SV40 early promoter minus the 72 bp repeats (9). This construct is transcriptionally silent in most cells, but expression can be stimulated by inserting enhancer fragments in the polylinker immediately upstream from the promoter. The plasmids we used are diagrammed at the right: pA0, containing the SV40 enhancer (9); pS3, containing the −2175 to +12 fragment of the MHC class II gene E^k_α (20); pSD212, bearing the −2175 to −1145 fragment of E_α; pX3, carrying only the −215 to +12 fragment of E_α; pXD21 and pXD301, bearing the same fragment as pX3 but with an X or Y box deletion (see reference 10 for description of the construction of these mutants). The −2175 to +12 region of E_α is depicted in the center of the figure: the thick line represents sequences coding for mRNA and the thin line signifies 5'-flanking sequences. Numbering begins at the major site of mRNA initiation (20). The Xho I and Bam HI sites are artificial, replacing the Acc I and Pvu I sites normally found at −215 and +12 (see reference 10 for details). Shaded boxes indicate the TATA box and the X and Y boxes, 14-base segments conserved in all MHC class II genes so far examined (see references 10, 11 for details).

Gel Retardation and Methylation Interference Assays. Nuclear extracts were prepared and gel retardation assays were performed as described. (10, 11). In short, a 1-M salt extract of nuclei was incubated with the ^{32}P-labeled test oligonucleotide and various amounts of poly[d(I-C)] competitor, the free DNA and DNA:protein complexes were separated on a polyacrylamide gel, and the resulting bands were revealed by autoradiography.

Methylation interference mapping has also been detailed (11). Briefly, ^{32}P-labeled double-stranded oligonucleotide was partially methylated and then incubated with nuclear extract in the presence of poly[d(I-C)]. Free and protein-bound DNA were separated, extracted, cleaved with piperidine (A+G), and electrophoresed on a sequencing gel.

Results and Discussion

RJ 2.2.5 Does Not Support Class II Gene Enhancer Activity. B cell-specific enhancer activity is associated with the 5'-flanking region of the murine class II gene, E_α. The cell type specificity and detailed localization of this enhancer are described elsewhere (Koch et al., submitted for publication); for this study, the relevant features are: (*a*) The E_α enhancer is cell type specific, operating in B cells but not in fibroblasts, epithelial cells, or murine T cells. (*b*) This enhancer was originally identified as a fragment that stimulates transcription from the enhancerless SV40 promoter in constructs like that diagrammed in Fig. 1. (*c*) Maximum stimulatory activity is associated

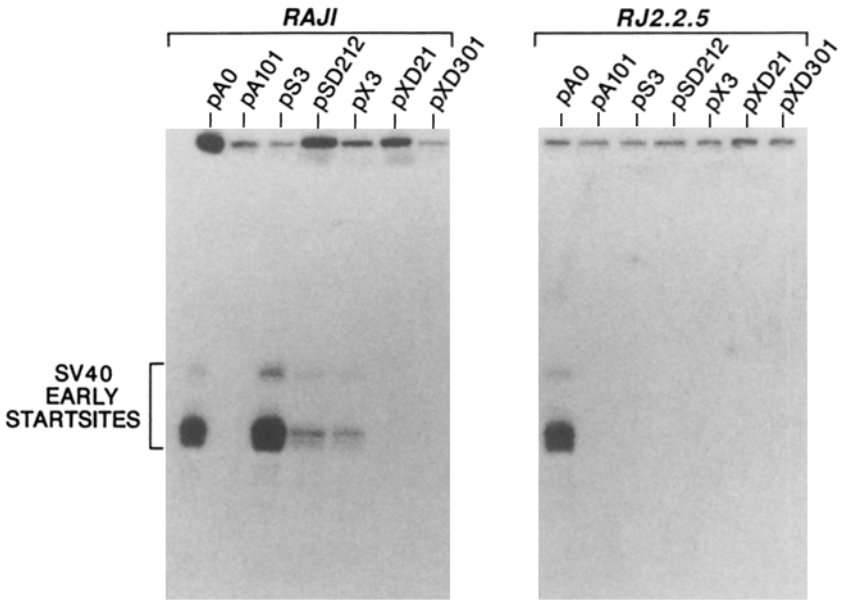


FIGURE 2. The E_α enhancer is inoperative in RJ 2.2.5. Plasmids described in Fig. 1 were transfected into Raji or RJ 2.2.5 cells. 2 d later, cytoplasmic RNA was isolated and subjected to S1 nuclease analysis to measure the amount of RNA initiated at the SV40 early startsites.

with the −2,175 to +12 fragment of the E_α gene, a map of which is also presented in Fig. 1. (*d*) If this fragment is bisected at position −1,145, both halves show enhancer activity. The promoter-proximal (right) half remains B cell specific; the promoter-distal (left) half functions in all cell types. (*e*) The −215 to +12 region contributes most (or all) of the activity associated with the promoter-proximal half of the enhancer. (*f*) Deletions of the X and Y boxes from the −215 to +12 fragment abrogate enhancer function. The X and Y boxes are conserved 14-base sequence elements located in the −50 to −100 region of all MHC class II genes so far examined. Both conserved segments are required for efficient and accurate transcription of the E_α gene in transgenic mice (10), and both are targets for sequence-specific DNA-binding proteins (10–13). The E_α gene has a second copy of the X and Y boxes, in reverse orientation, at about position −1300 (Fehling, J., unpublished results). (*g*) A human class II gene enhancer exhibiting some of these features has recently been described (14).

It was clearly of interest to compare the activity of the E_α enhancer in Raji and RJ 2.2.5, the mutant devoid of class II antigens. A series of plasmids were transfected into both cell lines, and the RNA transcribed from each construct was quantitated by S1 nuclease analysis. Fig. 2 shows that an enhancerless construct is not detectably expressed in Raji cells (pA101), but that the SV40 enhancer efficiently stimulates transcription (pA0). The complete E_α enhancer is even more powerful than the SV40 enhancer (pS3, −2,175 to +12). The individual halves of the E_α enhancer display stimulatory activity (pSD212, −2,175 to −1,145; pX3, −215 to +12), but the level of stimulation by each is less than that by the complete enhancer. Dele-

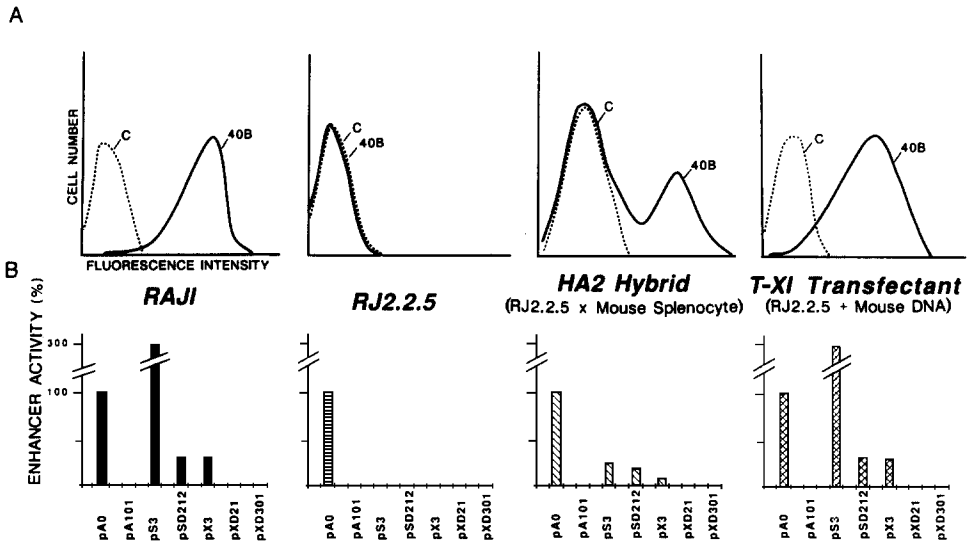


FIGURE 3. Correction of the deficiency in class II antigen expression restores the ability of RJ 2.2.5 to support E_α enhancer function. (*A*) Cytofluorimetric analysis of MHC class II antigen expression. HA2 derives from a fusion between RJ 2.2.5 cells and mouse splenocytes (4); the only mouse chromosomes present in these cells are 16 and 17 (5). The line T-XI is a secondary transfectant bearing mouse genomic DNA stably integrated into the genome of RJ 2.2.5. The staining of cells and analytical procedures on an ODAM ATC 3000 have been described (21). Cytofluorimetric profiles are shown for unstained cells (*dotted lines*) or for cells stained with 40B (*smooth lines*), a pan-class II antigen reagent. This mAb is an anti-mouse I-A and I-E reagent, strongly crossreactive with human class II antigens (22). Note that the HA2 line shows a biphasic distribution, due to the spontaneous segregation of mouse chromosome 16 leading to loss of class II gene expression (5). (*B*) Quantitation of enhancer activity. The four cell lines were transfected with the plasmids depicted in Fig. 1. 2 d later, cytoplasmic RNA was prepared and assayed by S1 nuclease mapping. Densitometric tracings of the bands representing S1-resistant hybrid were performed and the values were normalized, taking as 100% for each cell line the value obtained for RNA from pA0 transfected cells. The bar graphs are plots of averages from at least three independent experiments with two different plasmid preparations.

tion of the X (pXD21) or Y (pXD301) boxes in the −215 to +12 fragment abolishes enhancer function. In short, the E_α enhancer operates in Raji cells quite like it does in murine B cell lymphoma lines.

The results are quite different with RJ 2.2.5: none of the constructs carrying the E_α enhancer are detectably transcribed in this mutant cell line (Fig. 2). The same negative result has been observed in six separate experiments with two independent preparations of plasmid DNA. As might be expected, the enhancerless construct is not transcribed in RJ 2.2.5 cells (pA101), and the SV40 enhancer functions as effectively in this variant as it does in Raji (pA0).

RJ 2.2.5 Derivations Reexpressing Class II Antigens Support E_α Enhancer Activity. Expression of the MHC class II genes in RJ 2.2.5 cells can be restored by fusing them with mouse cells that express class II antigens (3–5) or by transfecting them with mouse genomic DNA (6). We wished to determine whether this restoration of endogenous class II gene activity correlated with a renewed ability to support E_α enhancer function.

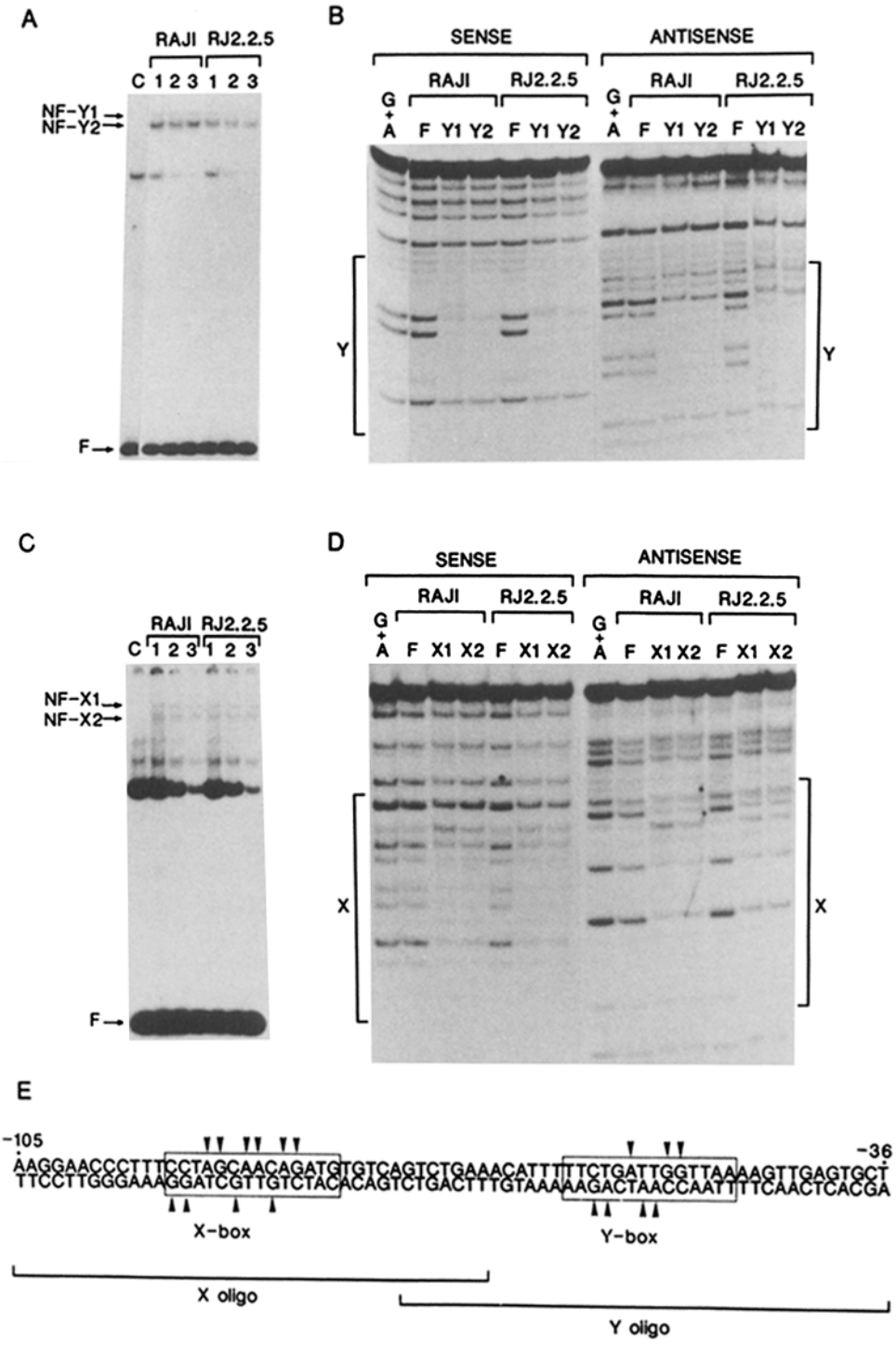
A cytofluorimetric analysis of human class II antigen expression on two such RJ 2.2.5 derivatives is presented in Fig. 3 *A*. The hybrid line A2-2.4c16 (HA2 for short) is derived from a fusion between RJ 2.2.5 cells and mouse splenocytes (4, 5). It carries only two mouse chromosomes, 16 and 17; chromosome 16 bears *aIr-1*, the locus that complements the RJ 2.2.5 defect (5). About 30% of the HA2 cells display human MHC antigens in this experiment; the others have probably lost chromosome 16, the karyotype of the cells being somewhat unstable. The line T-XIS5.7 (T-XI for short) is a secondary transfectant of RJ 2.2.5 that carries mouse genomic DNA incorporated into the genome (6). Essentially all of the T-XI cells express high levels of human class II antigens in this experiment.

To determine whether HA2 and T-XI support E_α enhancer activity, we transfected the aforementioned plasmids into them as well as into Raji and RJ 2.2.5 cells. The bar graphs in Fig. 3 *B* show transcription levels from the different constructs assayed in at least three experiments using two independent DNA preparations. The E_α enhancer functions in HA2 cells, although the level of transcriptional stimulation by the complete enhancer (pS3) is only 10% of that measured in Raji cells. A partial explanation for this lower value is that only some of the HA2 cells carry mouse chromosome 16. T-XI cells are also permissive for E_α enhancer activity; in this case, transcription driven by the complete enhancer is as efficient as in Raji cells.

RJ 2.2.5 Appears to Have Normal NF-X and NF-Y Proteins. RJ 2.2.5 cells thus appear to have a defect in some factor required for E_α enhancer function. We know that the X and Y boxes are critical for transcriptional enhancement (Figs. 2 and 3 *B*, and unpublished results), and that they are targets for the sequence-specific DNA-binding proteins NF-X and NF-Y (10–13). It was imperative, then, to determine whether these proteins are normal in RJ 2.2.5 cells.

The initial assay was the gel-retardation or band-shift assay. A 39-bp double-stranded oligonucleotide spanning the Y box was prepared (see Fig. 4 *E* for sequence) and 5′ end labeled with ^{32}P. The labeled oligonucleotide and unlabeled nonspecific competitor DNA were incubated with a 1-M salt extract of nuclei from either Raji or RJ 2.2.5 cells; lastly, the resultant DNA:protein complexes were separated from free DNA on a neutral polyacrylamide gel. Three retarded bands are evident in Fig. 4 *A*. The most rapidly migrating species is probably due to nonspecific protein:DNA interactions because it is also observed with a control oligonucleotide bearing random sequence in place of the Y box (lane *C*), as well as with several unrelated oligos (not shown). NF-Y2 is probably very similar (or identical) to a CCAAT box-binding protein termed NF-Y that we identified in nuclear extracts from murine cell lines (10–12). NF-Y1 is not found in murine extracts but occurs in all human extracts so far examined (Raji, RJ 2.2.5, HeLa, MCF-7). These three retarded bands appear identical with Raji and RJ 2.2.5 extracts, and so we are led to conclude that there must be no gross differences in NF-Y.

A more sensitive assay is provided by methylation interference mapping. A gel retardation experiment was conducted as described above except that one strand of the Y oligonucleotide had been partially methylated. After electrophoresis, DNA from the NF-Y1, NF-Y2, and F bands were extracted, cleaved at methylated bases, and displayed on a sequencing gel. Comparison of the patterns obtained with bound vs. free DNA should reveal protein contact sites; if methylation of a particular site inhibits protein binding, that site will not appear as a band, but rather as a hole.


A
RAJI RJ2.2.5
C 1 2 3 1 2 3
NF-Y1
NF-Y2
F
B
SENSE
ANTISENSE
RAJI RJ2.2.5
RAJI RJ2.2.5
G + A F Y1 Y2 F Y1 Y2
G + A F Y1 Y2 F Y1 Y2
Y
Y
C
RAJI RJ2.2.5
C 1 2 3 1 2 3
NF-X1
NF-X2
F
D
SENSE
ANTISENSE
RAJI RJ2.2.5
RAJI RJ2.2.5
G + A F X1 X2 F X1 X2
G + A F X1 X2 F X1 X2
X
X
E
-105
-36
AAGGAACCCTTTCCTAGCAACAGATGTGTCAGTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAGTGCT
TTCCTTGGGAAAGGATCGTTGTCTACACAGTCTGACTTTGTAAAAAGACTAACCAATTTTCAACTCACGA
X-box
Y-box
X oligo
Y oligo

Two points are clear from Fig. 4 *B*. First, Y1 and Y2 seem to make the same contacts on the Y box; these are indicated on the sequence in *E*. Second, and more relevant for this study, Raji and RJ 2.2.5 proteins show essentially identical contacts.

Similarly, when a ^{32}P-labeled X box oligonucleotide is incubated with nuclear extract from either Raji or the variant, two specific DNA:protein complexes are detected in the gel retardation assay (Fig. 4 *C*). Again, the more rapidly migrating bands are probably due to nonspecific interactions (compare with binding to the control oligonucleotide in lane *C*). Methylation interference mapping shows that NF-X1 and NF-X2 contact a single set of bases on the X box, and that these contacts are the same with extracts from Raji or RJ 2.2.5 cells (Fig. 4, *D* and *E*).

Thus, by these criteria the NF-X and NF-Y proteins do not differ between the two cell lines. The NF-Ys are also indistinguishable on the basis of apparent molecular weight of the intact protein and of a protease-resistant DNA-binding core (data not shown). We are left with the conclusion that any defect in NF-X or NF-Y must be quite subtle, or that these proteins are normal in RJ 2.2.5 cells. Miwa et al. (13) have also recently shown that the Raji and RJ 2.2.5 proteins binding to X and Y box oligonucleotides are similar in gel retardation assays, but it is not possible to determine which of the proteins they detect (if any) correspond to NF-X and NF-Y.

Conclusions. We have identified the function of a regulatory factor that is defective in RJ 2.2.5 cells; this factor is absolutely required for the activity of an MHC class II gene enhancer. Interestingly, the E_α enhancer comprises two components, and neither is functional in RJ 2.2.5. When class II gene expression is restored in RJ 2.2.5 derivatives, the enhancer becomes active once again, and the two halves are activated concomitantly. Altogether, these results suggest that the two halves of the enhancer probably operate by a similar mechanism, using at least some of the same factors.

This conclusion may not be surprising when one considers the architecture of the E_α 5′-flanking region; there is an X and Y box in the −50 to −100 region and another pair, in reverse orientation, at around position −1,300; other sequences ad-

FIGURE 4. The NF-X and NF-Y proteins of Raji and RJ 2.2.5 are indistinguishable. (*A* and *C*) Gel retardation assays. For Y, a fixed amount of extract (1 μg protein) was incubated with a 5′ end-labeled double-stranded oligonucleotide and increasing amounts of poly[d-(I-C)] competitor. For X, the nuclear extract was first precipitated with 50% ammonium sulphate and concentrated fivefold; 7.5 μg of protein was used in the reaction. Uncomplexed and protein-complexed DNAs were resolved on a 5% polyacrylamide gel. The test oligonucleotides span the X (*C*) or Y (*A*) boxes; the actual sequences are indicated in *E*. To control for nonspecific binding to the target DNA, control oligonucleotides were made that have random sequences replacing the X or Y box but have the same 5′- and 3′-flanking sequences. Results with these oligonucleotides are shown in lanes *c* of panels *A* and *C*. Amounts of poly[d(I-C)] in lanes *1–3* were 100, 300, and 1,000 ng in both panels. Y1 and Y2 represent specific DNA/protein complexes formed on the Y oligonucleotide; X1 and X2 represent specific complexes formed on the X oligonucleotide; F signifies uncomplexed DNA.

(*B* and *D*) Methylation interference mapping. Binding reactions and gel resolution of the DNA/protein complexes were essentially as above, except that the reactions were scaled up and the X and Y oligonucleotides were partially methylated with dimethylsulphate before purification. The experiment was done with DNA 5′-end-labeled either on the sense or antisense strand. Uncomplexed (F) or protein-complexed (Y1, Y2, X1, X2) DNA were eluted from the gel, cleaved by piperidine (G + A) and displayed on a denaturing gel. *B* shows the pattern for the Y oligonucleotide and *D* shows the pattern for the X oligonucleotide. The bases whose methylation interferes with binding are indicated in *E*.

jacent to the X and Y boxes are also duplicated. We know that the X and Y boxes are very important to the promoter—proximal half because deleting them from the −215 to +12 fragment abolishes enhancer activity (Figs. 2, and 3; Koch et al., submitted for publication). Yet they are not solely responsible for enhancer function because, alone, they do not stimulate transcription that efficiently (Koch et al., submitted for publication). The X-Y complex in the promoter—distal half also seems to play some role in enhancer activity according to transfection studies but even more certainly, other sequence motifs are critical (Koch et al., submitted for publication). Nevertheless, the X and Y boxes remain the sequence elements the most strikingly similar between the E_α promoter-proximal and promoter-distal halves of the enhancer.

Some enhancer factor seems to be aberrant in the mutant; yet, the most likely candidates, NF-X and NF-Y, appear normal. It is possible that the defect in RJ 2.2.5 is a subtle mutation of NF-X or NF-Y that does not affect their DNA-binding characteristics; is the alteration of another DNA-binding protein; is the mutation of a factor that does not bind to DNA but interacts with NF-X, NF-Y, or another DNA-binding protein; or is an incorrect post-translational modification of any of these proteins. One could also envisage more remote mechanisms: local disruptions of chromatin structure, aberrant scaffold attachment sites etcetera. In fact, such a mechanism may be implicated by the recent finding that introduction of the *ras* oncogene into RJ 2.2.5 cells at least partially restores class II gene expression (15). Whatever the exact defect, it is not a generic one, affecting the activity of all enhancers. Immunoglobulin gene expression is not extinguished, and the SV40 enhancer operates quite efficiently in RJ 2.2.5 (Fig. 2). In addition, the mutant cells thrive in culture, exhibiting a normal doubling time.

RJ 2.2.5 represents the first mammalian cell mutant with a documented defect in an enhancer factor (excluding steroid hormone receptors). Atchison and Perry (16) have recently described a plasmacytoma that exhibits a defect in an immunoglobulin enhancer-binding factor, but it is not excluded that this line represents a normal stage of differentiation. The RJ 2.2.5 line should prove extremely useful in experiments designed to unravel the mechanism of enhancer function. Complementation analysis, by DNA transfection or by fusion with other class II deficient cell lines (17–19), could lead to an understanding of the genetics of MHC class II gene regulation at a level currently possible only with yeast regulatory systems.

Summary

RJ 2.2.5 is an immunoselected mutant of the Burkitt lymphoma line Raji. It fails to display MHC class II antigens at the cell surface due to a transcriptional defect. We have identified the function of a regulatory factor that is defective in RJ 2.2.5 cells; this factor is absolutely required for the activity of an MHC class II gene enhancer.

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