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# Recessive Genetic Deregulation Abrogates c-*myc* Suppression by Interferon and Is Implicated in Oncogenesis

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**In a previous study we demonstrated that many hematopoietic tumor cells are resistant to the inhibitory effects that interferon exerts on c-*myc* mRNA expression without losing other receptor-mediated intracellular responses (M. Einat, D. Resnitzky, and A. Kimchi, Nature [London] 313:597–600). We report here that this partial resistance was overridden in two independent stable somatic cell hybrids prepared by fusion between sensitive and resistant cells. The c-*myc* mRNA transcribed from the active allele of the resistant parent cell was reduced by interferon within the context of the cell hybrid. It was therefore concluded that changes in the *cis*-acting sequences of c-*myc* were not involved in this type of relaxed regulation and that resistance resulted rather from inactivation or loss of postreceptor elements which operate in *trans*. The growth-stimulating effect that this genetic deregulation might have on cells was tested in experimental systems of cell differentiation in which an autocrine interferon is produced. For that purpose we isolated variant clones of M1 myeloid cells which were partially resistant to alpha and beta interferons and tested their growth behavior during in vitro-induced differentiation. The resistant clones displayed higher proliferative activity on days 2 and 3 of differentiation than did the sensitive clones, which stopped proliferating. The loss of c-*myc* responses to the self-produced interferon disrupted the normal cessation of growth during differentiation and therefore might lead cells along the pathway of neoplasia.**

The active expression of the c-*myc* nuclear oncogene is characteristic of normal proliferating cells and is directly modulated by growth factors (18). Expression of c-*myc* is reduced when cells enter the quiescent nonproliferative state of the cell cycle, as shown in various in vitro systems of terminal differentiation in which the cells gradually stop proliferating (7, 10, 15, 25, 40). The implication of c-*myc* activation in oncogenesis has been widely studied during the past few years (20). Unlike other oncogenes which become activated by structural alterations of the protein product, the deregulation of c-*myc* in many tumors involves disruption of the control elements that enable the normal gene to be switched off (20). Therefore, to understand the molecular basis of c-*myc* activation in tumor cells it is important to analyze how the gene is normally suppressed in differentiated cells.

We previously reported that a self-secreted beta interferon (IFN-β) is utilized by differentiating hematopoietic cells to autoregulate c-*myc* suppression (13, 33, 42). We based this conclusion on the finding that antiserum to IFN-β partially abrogates the typical reduction of c-*myc* mRNA during the in vitro-induced differentiation of M1 myeloid cells (33). Exogenous IFN-α, -β, or -γ reduces c-*myc* mRNA levels in some target cells (6, 8, 10, 11, 17, 19, 22, 41) but not in many hematopoietic tumor cell lines which express a resistant phenotype and fail to respond to IFN by c-*myc* inhibition (10). In light of the basic involvement of autocrine IFN in c-*myc* regulation it became important to investigate the molecular basis of the loss of sensitivity to IFN in tumor cells and to study how this specific deregulation might affect the process of terminal differentiation.

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In this study we first analyzed the response to IFN of somatic cell hybrids between sensitive and resistant Burkitt lymphoma cells. We found that the IFN-mediated transcriptional inhibition of c-*myc* was dominant over the nonresponsiveness of resistant cells and that changes in *cis*-acting regulatory elements of c-*myc* were not involved in the relaxed regulation. By isolating IFN-resistant (IFN$^r$) cell variants from the M1 myeloid cell line we demonstrated that the loss of sensitivity to IFN changed the growth kinetics of cells during in vitro-induced differentiation. Genetic deregulation which disrupts the suppression of c-*myc* by IFN results from the loss or inactivation of elements which operate in *trans* and interferes with the normal growth arrest of differentiating cells.

## MATERIALS AND METHODS

**Cells.** The Burkitt lymphoma cell lines were grown in RPMI 1640 (GIBCO Laboratories) supplemented with 10% heat-inactivated fetal calf serum (BioLab) and penicillin-streptomycin (50 μg/ml) in 5% $CO_2$–95% air at 37°C. The preparation and characterization of the two somatic cell hybrids were previously described in detail (21, 43). The hybrid DITRUD was derived by fusing a 6-thioguanine-resistant variant of Daudi cells (DIT) with a thymidine kinase-deficient variant of Raji cells (RUD). The DUT cells which we used in these experiments as representative of the Daudi parent cells are ouabain-resistant variants of the DIT cells used for the fusion process. The hybrid RUDPUT was derived by fusing RUD cells with PUT, a 6-thioguanine- and ouabain-resistant double mutant of P3HR1. The modal chromosome number of both hybrids was as expected from the summation of the parental cell modal numbers.

Mouse M1 myeloid cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. When needed, 12% (vol/vol) mouse lung-conditioned medium was added to induce the differentiation towards granulocytes and macrophages as previously described (33). The lung-conditioned medium was prepared and tested for the absence of IFN activity as previously described (33). Lysozyme activity was determined in 0.5-ml samples of growth medium by monitoring the decrease in the turbidity (540 nm) of a suspension of *Micrococcus lysodeikticus* with hen egg white lysozyme as a standard (Sigma Chemical Co.). Results are expressed as the total amount of lysozyme in unit equivalents secreted by $5 \times 10^6$ cells. Randomly isolated clones of M1 myeloid cells were used for these studies (13 clones). From one of these clones (the parental clone), the IFN$^r$ cell variants were isolated as follows. Exponentially growing cells ($2 \times 10^5$ cells per ml, $10^7$ cells) were continuously cultured in the presence of mouse IFN-α plus IFN-β (400 U/ml). Growth-arrested cultures were fed with fresh medium containing IFN twice a week and were kept for 4 to 5 weeks until resistant cells capable of growing in the presence of IFN appeared. The cells in this culture were then recloned, and 23 IFN$^r$ clones were collected and further tested. The frequency of growth-resistant variants was calculated to be around $10^{-5}$. These cells were continuously maintained with 400 U of mouse IFN-α plus IFN-β per ml; before the experiment they were grown for 10 to 20 generations without IFN. The resistant phenotype was stable and was not lost even after a few months of growth in the absence of IFN. Cell numbers were monitored with a Coulter counter. 2′,5′-oligoadenylate (2′,5′-oligo A) synthetase activity was measured in crude cell extracts as previously described (10).

**Quick cell blot hybridization.** Quick cell blot hybridization was done with guanidine hydrochloride solubilization of whole cells and selective precipitation of RNA as described by Cheley and Anderson (4). Samples corresponding to $2 \times 10^6$, $1 \times 10^6$, and $5 \times 10^5$ cells were applied to nitrocellulose filters, mounted inside a filtration apparatus, and hybridized to a nick-translated c-*myc* probe. The hybridization and washing conditions of the filters were as previously described (10, 41).

**RNA blot analysis.** Total RNA was isolated by the LiCl method from $1 \times 10^8$ to $5 \times 10^8$ cells as previously described (10). Selected poly(A)$^+$ mRNA obtained by purification on oligo(dT)-cellulose chromatography was subjected to electrophoresis through 1% agarose gels in 6% formaldehyde. The RNA was transferred to nitrocellulose sheets and hybridized to a nick-translated c-*myc* or actin probe as previously described (41).

**Nuclear runoff assays.** The analysis of c-*myc* gene transcription in isolated nuclei was performed as described elsewhere (10). Briefly, nascent RNA chains were allowed to elongate in the presence of [α-$^{32}$P]UTP. Nuclei were isolated from $10^8$ cells as previously described (10), and $4 \times 10^7$ nuclei were incubated for 15 min at 25°C. The $^{32}$P-labeled nuclear RNA was subjected to DNase I treatment, phenol-choroform extracted, and filtered through Sephadex G-50. Equivalent amounts of trichloroacetic acid-precipitable $^{32}$P-labeled RNA ($10^7$ cpm) were hybridized to nitrocellulose filters on which 10-μg samples of recombinant pBR322 plasmids containing the 1.4-kilobase (kb) *Cla*I-*Eco*RI genomic fragment of human c-*myc* (third exon), actin cDNA plasmids, or pBR322 plasmids alone were immobilized as described before (41). The $^{32}$P-labeled RNA bound to filters after washing and treatment with RNase A was visualized by autoradiography.

**Southern blot analysis.** Genomic DNA was extracted from cells and digested with restriction enzymes, and 10-μg samples were subjected to gel electrophoresis and to Southern transfer. The hybridization conditions were as follows: 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin, 50 mM phosphate buffer (pH 6.5), 10% dextran sulfate, and sonicated salmon sperm DNA (75 μg/ml) containing $2 \times 10^6$ cpm of nick-translated $^{32}$P-labeled probe per ml (specific activity, $2 \times 10^8$ cpm/μg). After hybridization for 24 h at 42°C, filters were washed at 70°C with 0.1× SSC–0.1% sodium dodecyl sulfate–0.1% sodium $PP_i$ and autoradiographed at −70°C for 12 h. Lambda phage DNA cut with *Hin*dIII was run in the same gel to determine the restriction fragment sizes.

**RNase protection mapping.** The RNA used for these experiments was extracted by the LiCl method, and either 2 μg of selected poly(A)$^+$ RNA or 20 μg of total RNA was analyzed. The hybridization probe was produced by the in vitro transcription of a plasmid which contains the *Xho*I-*Xba*I genomic fragment of c-*myc* inserted into the *Sal*I-*Xba*I sites of the pGEM-1 riboprobe vector (Promega Biotech) downstream of the T7 phage promoter element. Uniformly labeled antisense RNA chains were synthesized in vitro following linearization of the vector with T7 RNA polymerase, and the reaction was stopped by DNase treatment and phenol-chloroform extraction. The labeled RNA products were separated on preparative polyacrylamide gels, and full-length RNA transcripts were electroeluted from the gel. Samples of $^{32}$P-RNA probe ($5 \times 10^5$ cpm) were mixed with the poly(A)$^+$ or total RNA at 85°C for 5 min, followed by incubation at 55°C for 16 h. The hybridization conditions were as follows: 80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.7), 0.4 M NaCl, and 1 mM EDTA. Unhybridized single-stranded RNA was digested with RNase A (40 μg/ml; Sigma) and RNase $T_1$ (2 μg/ml; Boehringer Mannheim Biochemicals) for 60 min at 37°C. Following proteinase K treatment (150 μg/ml) and phenol-chloroform extraction, the double-stranded $^{32}$P-RNA-RNA duplexes were heat denatured and analyzed on denaturing gels (6% polyacrylamide–8 M urea). $^{32}$P-end-labeled pBR322 DNA fragments cut with *Hin*fI were used as molecular weight markers.

**Materials.** Human IFN-α ($5 \times 10^8$ U/mg) was purified to homogeneity by monoclonal antibody columns as described elsewhere (31). Mouse IFN (IFN-α plus IFN-β) was purchased from Lee Biomolecular ($2 \times 10^8$ U/mg). The human c-*myc* genomic probe (*Cla*I-*Eco*RI fragment containing the third exon and 3′-flanking sequences) was previously described (10). The *Xho*I-*Xba*I genomic fragment of human c-*myc* containing the first exon and part of the first intron was derived from the 12.7-kb *Eco*RI c-*myc* genomic fragment (provided by P. Leder). The murine c-*myc* genomic probe was a 3.7-kb *Bam*HI-*Hin*dIII fragment carrying the second and third exons and part of the first intron as described elsewhere (11). The actin probe was previously described (11).

## RESULTS

**Reduction of c-*myc* expression by IFN is a dominant property in somatic cell hybrids between sensitive and resistant Burkitt lymphoma cells.** In Burkitt lymphoma cells the c-*myc*

gene on normal chromosome 8 is silent, while its allele which has been translocated to the immunoglobulin gene locus is constitutively active (27). Despite this deregulation it has been reported that c-*myc* mRNA steady-state levels can be reduced by exogenous IFN-α or IFN-β in Daudi Burkitt lymphoma cells (6, 8, 10, 17). Another Burkitt lymphoma cell line, Namalwa, failed to respond to IFN by c-*myc* inhibition but did respond normally to the other effects of IFN (19). We recently studied several additional Burkitt lymphoma cell lines and found that only two of nine cell lines were sensitive to IFN (data not shown). To gain insight into the molecular mechanisms which lead to a lack of response of c-*myc* to IFN in resistant cells, we analyzed somatic cell hybrids prepared between resistant and sensitive Burkitt lymphoma cells. We chose as target cells three different Burkitt cell lines, Daudi, P3HR1, and Raji, which all carry a t(8:14) type translocation and differ from each other in the positions of the breakpoints on both chromosomes (27). Somatic cell hybrids prepared by fusion of Raji cells with either Daudi or P3HR1 cells were previously obtained and characterized by means of chromosome, isozyme, and class I histocompatibility antigen surface markers (21, 43). The parental Daudi-, Raji-, and P3HR1-derived cell lines having different selective markers (termed DUT, RUD, and PUT, respectively) and the corresponding cell hybrids (DITRUD and RUDPUT) were all analyzed simultaneously for their response to IFN.

The effect of IFN on c-*myc* mRNA levels was measured by two different techniques. In Fig. 1A, quantitation was performed by the quick cell blot hybridization assay, which yields values on the basis of cell number (4). Twofold dilutions of crude RNA preparations extracted from $2 \times 10^6$ cells before and after treatment with IFN-α (100 U/ml) were spotted on nitrocellulose filters and hybridized to nick-translated c-*myc* genomic fragments. In parental DUT and PUT cells, IFN-α reduced four- to sixfold the c-*myc* mRNA levels per cell. In contrast, a similar treatment of parental RUD cells with IFN-α did not reduce but rather stimulated two- to threefold the c-*myc* mRNA levels per cell (Fig. 1A). DUT and PUT cells were therefore sensitive to IFN. However, RUD cells,which are the common parental cells in both hybrids, had a resistant phenotype with regard to c-*myc* expression, like many other leukemic cells which we have tested (10) and as expected from the well-known growth resistance of Raji cells to IFN (24). Interestingly, we found that each of the cell hybrids, DITRUD and RUDPUT, had a sensitive phenotype and responded to IFN-α by a reduction of c-*myc* mRNA levels per cell (Fig. 1A). Similar results were obtained when the c-*myc* mRNA transcripts were analyzed on Northern (RNA) blots (Fig. 1B). The steady-state levels of the 2.4-kb c-*myc* transcripts in the DITRUD cell hybrid were reduced following treatment with IFN to the same extent as the reduction observed in the sensitive parental DUT cells, unlike the RUD cells (Fig. 1B). In the RUDPUT cell hybrid, an additional longer (4.2-kb) c-*myc* mRNA, probably representing unspliced transcripts, was detected. The latter originated from the sensitive PUT cells and was reduced by IFN within the cellular context of the hybrid to the same extent as the well-characterized 2.4-kb c-*myc* transcripts (Fig. 1B). The β-actin mRNA levels did not change in response to the IFN treatment, suggesting a selective effect on c-*myc* expression (Fig. 1B).

These results indicate that the property of c-*myc* inhibition by IFN is dominant and overrides resistance in two stable somatic cell hybrids. It should be emphasized that the resistance of RUD cells was not due to inactive cell surface receptors, since these cells responded normally to IFN with respect to the induction of 2′,5′-oligo A synthetase, the product of one of the IFN-induced genes (29). The synthetase activity was activated in a similar manner in all three parental cells in response to an increasing dosage of IFN-α (Table 1). The difference between these cell lines therefore resides at the postreceptor level.

TABLE 1. Induction of 2′,5′-oligo A synthetase by IFN-α in Burkitt lymphoma cells[a]

| IFN (U/ml) | 2′,5′-oligo A synthetase activity in the following cells (cpm/10 μg of protein): | | |
|---|---|---|---|
| | PUT | RUD | DUT |
| 0 | 22,000 | 4,600 | 13,000 |
| 1 | 65,200 | 12,000 | |
| 10 | 106,700 | 61,631 | 50,000 |
| 100 | 153,600 | 96,600 | 130,000 |
| 1,000 | 123,000 | 102,500 | 120,000 |

[a] Exponentially growing cells were treated with IFN-α; 24 h later cell extracts were prepared and samples were tested for 2′,5′-oligo A synthetase activity (10).

The reduction by IFN of c-*myc* mRNA steady-state levels detected in sensitive parental cells and subsequently in somatic cell hybrids could take place at transcriptional or posttranscriptional levels of regulation. It should be mentioned that in previous studies which analyzed the effect of IFN on Daudi Burkitt lymphoma cells, inhibition at both levels has been reported, possibly reflecting differences in the Daudi cell lines tested (10, 22). Nuclear runoff experiments were therefore performed on one of these cell hybrids, RUDPUT, before and after treatment with IFN-α (100 U/ml). We found that the bulk rate of RNA production by isolated nuclei was unchanged following exposure of RUDPUT cells to IFN-α (data not shown). However, measurements of in vitro-transcribed c-*myc* mRNA by hybridization to the third exon of the gene revealed a significant reduction by IFN, while β-actin transcription was not affected (Fig. 1C). IFN therefore selectively affects the transcriptional activity of the c-*myc* gene in the RUDPUT cell hybrid, consistent with our previous reports on sensitive Daudi Burkitt lymphoma cells (10). It should be emphasized, however, that we have not yet distinguished in these experiments between reduction of transcriptional initiation or elongation (2).

**Expression of the Raji c-*myc* allele is reduced by IFN within the cellular context of the hybrid.** It has been reported that the breakpoint on chromosome 8 in Raji cells occurs 1.4 kb upstream of the promoter region of c-*myc*, thus removing two main DNase I-hypersensitive sites (I and IIa) from the 5′-flanking sequences of the c-*myc* gene (9). In contrast, all five DNase I-hypersensitive sites, initially described by Siebenlist et al. (36), remain intact in Daudi and P3HR1 cells, since the breakpoint occurs more than 20 kb upstream of the start point of c-*myc* (5). The three parental cell lines also differ in the position and frequency of somatic mutations, which are spread mostly within the first noncoding exon of the c-*myc* gene (32, 37). In light of these structural differences, it is possible that the resistance of Raji cells to IFN results from changes in the *cis*-acting regulatory elements of the gene. Along this line, the sensitive phenotype of the cell hybrids tested above could simply result from the fact that the Raji translocated c-*myc* allele is absent from or not expressed in these cells. Alternatively, disruption of another gene which mediates c-*myc* suppression by IFN could generate resistance. Inactivation or the complete absence of

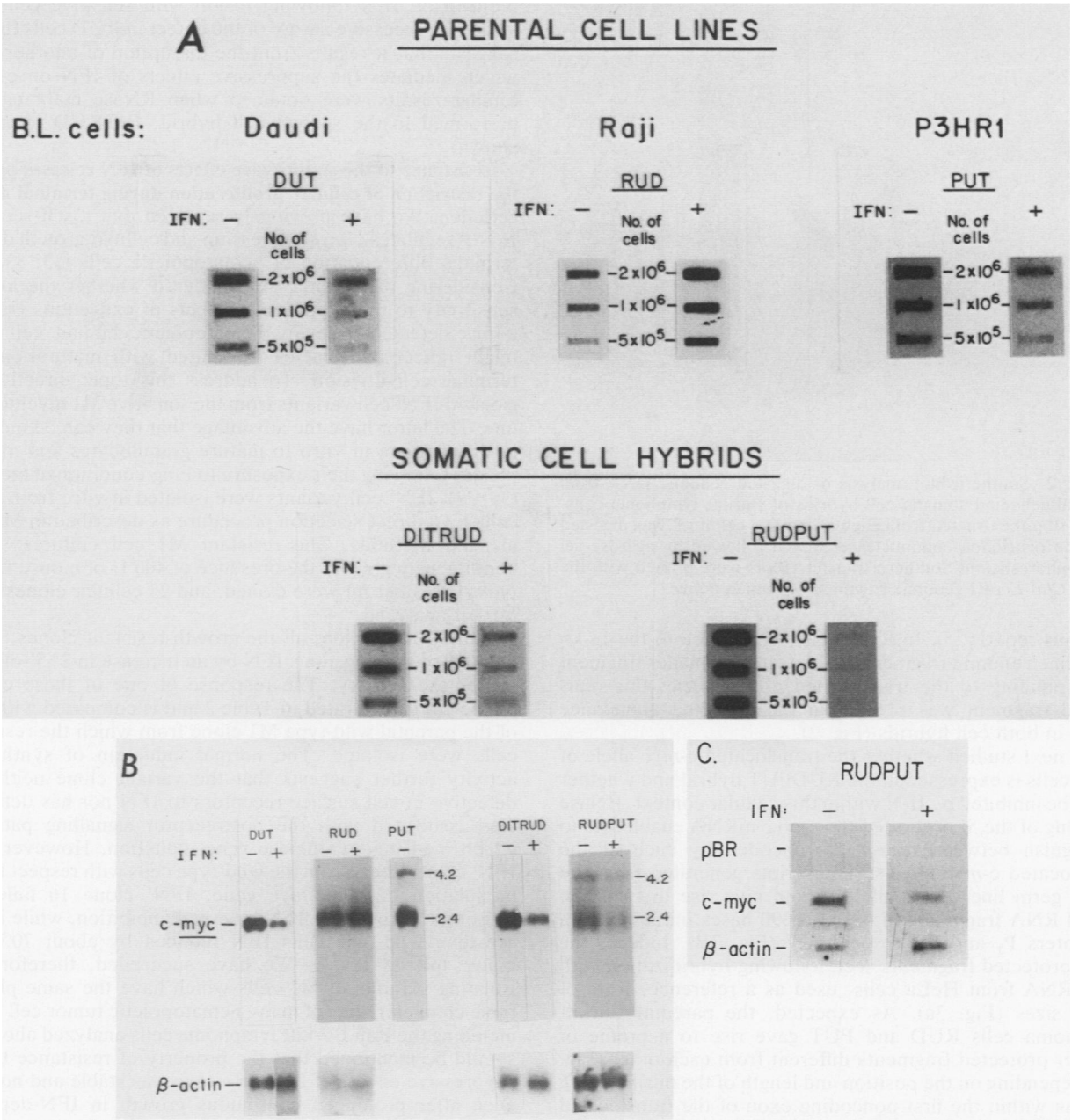


FIG. 1. Effect of IFN-α on c-*myc* mRNA expression in somatic cell hybrids between sensitive and resistant Burkitt lymphoma (B.L.) cells. The different cell lines were treated for 48 h (A) or 24 h (B and C) with IFN-α (100 U/ml) during the exponential growth phase and further tested for c-*myc* mRNA expression as follows. (A) Quick cell blot hybridization. Twofold successive dilutions of crude RNA preparations corresponding to the indicated cell numbers were spotted on nitrocellulose filters and hybridized to a nick-translated genomic fragment of the human c-*myc* gene as described in Materials and Methods. (B) Northern blot analysis of c-*myc* mRNA. Selected poly(A)$^+$ RNAs were separated on gels, Northern blotted, and hybridized to the c-*myc* genomic probe. The blots were rehybridized with the actin cDNA probe. Numbers at right are in kilobases. (C) Measurements of c-*myc* transcription in nuclear runoff assays. Equal amounts of trichloroacetic acid-precipitable $^{32}$P-RNA ($10^7$ cpm) were hybridized to recombinant pBR322 plasmids containing the third exon of human c-*myc*, actin cDNA plasmids, and control pBR322 plasmids, immobilized on nitrocellulose filters.

such a putative gene in Raji cells should lead to a recessive deregulation which could be overridden in the cell hybrid by the normal allele of the gene contributed by the sensitive cell. According to the second possibility, the expression of the Raji translocated c-*myc* allele should be reduced within the cellular context of the hybrid despite the structural alterations mentioned above. To distinguish between the two possibilities, we analyzed the two cell hybrids for the presence and expression of the translocated c-*myc* allele of the resistant Raji cells.

Southern blot analysis of the *Eco*RI digest of genomic DNA revealed that the RUD translocated c-*myc* allele was present in both cell hybrids, RUDPUT and DITRUD. In DUT and PUT only one restricted genomic fragment hybridized to the c-*myc* probe (Fig. 2), corresponding to both the germ line and the translocated c-*myc* alleles, consistent with

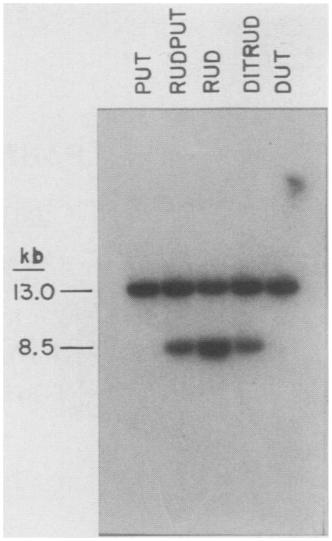


FIG. 2. Southern blot analysis of digested genomic DNA from parental cells and somatic cell hybrids of Burkitt lymphoma cells. DNA (10 μg) extracted from each of the five cell lines was digested with the restriction endonuclease *Eco*RI, followed by agarose gel electrophoresis and Southern transfer. Blots were probed with the 1.4-kb *Cla*I-*Eco*RI genomic fragment of human c-*myc*.

previous reports (5). In RUD cells, in addition to the 13-kb germ line genomic fragment, we detected a smaller fragment corresponding to the translocated c-*myc* allele; this rearranged fragment was present in the expected abundance (25%) in both cell hybrids (Fig. 2).

We next studied whether the translocated c-*myc* allele of RUD cells is expressed in the RUDPUT hybrid and whether it can be inhibited by IFN within this cellular context. RNase mapping of the 5′ portion of the c-*myc* mRNA enabled us to distinguish between transcripts encoded by each of the translocated c-*myc* alleles. Transcripts generated from the intact germ line c-*myc* allele should give rise to two protected RNA fragments of 485 and 390 bases initiating from promoters $P_1$ and $P_2$, respectively (Fig. 3B). Indeed, the main protected fragments seen following hybridization with total RNA from HeLa cells, used as a reference, were of those sizes (Fig. 3a). As expected, the parental Burkitt lymphoma cells RUD and PUT gave rise to a profile of smaller protected fragments different from each other (Fig. 3a), depending on the position and length of the mismatching regions within the first noncoding exon of the translocated allele. Full-length fragments were not detected in these cells, consistent with previous reports showing that the germ line c-*myc* allele is silent in Daudi and P3HR1 cells and suppressed to various levels in Raji sublines available in different laboratories (32, 34, 39). Figure 3a clearly illustrates that in the RUDPUT cell hybrid, the pattern of protected fragments consisted of a combination of both, those contributed by Raji cells (marked by arrows; 210-, 180-, 175-, and 120-base fragments) and those contributed by PUT cells (marked by small lines). Both translocated c-*myc* alleles originating from the resistant and sensitive parental cells were therefore expressed in the stable hybrid. Moreover, the fragments protected by the c-*myc* allele of RUD cells were reduced by IFN in the RUDPUT hybrid to the same extent as those protected by the c-*myc* allele of PUT cells. These results rule out the possibility that the relaxed regulation of c-*myc* in RUD cells resulted from structural changes in the translocated c-*myc* allele, since its expression could be reduced by IFN following fusion with sensitive cells. Instead, the recessive nature of the defect in RUD cells further suggests that it results from the disruption of another gene which mediates the suppressive effects of IFN on c-*myc*. Similar results were obtained when RNase mapping was performed in the second cell hybrid, DITRUD (data not shown).

**Resistance to the suppressive effects of IFN releases part of the restriction of cellular proliferation during terminal differentiation.** We have previously reported that a self-secreted IFN-β regulates c-*myc* expression and cellular growth during terminal differentiation of hematopoietic cells (13, 33, 42). Considering these data, we wondered whether the loss of sensitivity to the suppressive effects of exogenous IFN on c-*myc* detected in many hematopoietic human cell lines might reflect deficiencies associated with maturation and terminal cell division. To address this topic directly, we isolated $IFN^r$ cell variants from the sensitive M1 myeloid cell line. The latter have the advantage that they can be induced to differentiate in vitro to mature granulocytes and macrophages following their exposure to lung-conditioned medium (30, 33). $IFN^r$ cell variants were isolated in vitro from these cells by a direct selection procedure as described in Materials and Methods. The resistant M1 cell cultures which continuously grew in the presence of 400 U of mouse IFN-α plus IFN-β per ml were cloned, and 23 cellular clones were further analyzed.

Without exception, all the growth-resistant clones tested responded to exogenous IFN by an increase in 2′,5′-oligo A synthetase activity. The response of one of these clones (clone 16) is illustrated in Table 2 and is compared with that of the parental wild-type M1 clone from which the resistant cells were isolated. The normal induction of synthetase activity further suggests that the variant clone neither is defective in cell surface receptors to IFN nor has deficiencies associated with the postreceptor signalling pathway which mediates synthetase gene induction. However, this $IFN^r$ clone differs from the wild-type cells with respect to the modulation of the c-*myc* gene. $IFN^r$ clone 16 failed to respond to exogenous IFN by c-*myc* inhibition, while in the sensitive wild-type cells IFN reduced by about 70% the c-*myc* mRNA levels. We have succeeded, therefore, in isolating variants of M1 cells which have the same phenotypic characteristics of many hematopoietic tumor cell lines, including the Raji Burkitt lymphoma cells analyzed above. It should be mentioned that the property of resistance to the suppressive effects of IFN on c-*myc* was stable and not lost even after prolonged continuous growth in IFN-depleted medium (data not shown).

The variant cells were induced to differentiate by lung-conditioned medium, and several parameters were measured (Table 2 and Fig. 4). Lysozyme activity in the growth medium represents a well-defined differentiation marker in myeloid cells, while an increase in 2′,5′-oligo A synthetase activity in crude cell extracts reflects the production of and response to autocrine IFN in this system (33). $IFN^r$ clone 16 responded like wild-type cells to the differentiation stimulus with respect to lysozyme induction and production of autocrine IFN. However, c-*myc* suppression at day 3 of differentiation was impaired in the IFN-resistant cells, and only a partial reduction of c-*myc* mRNA (about 50%) was detected (Table 2). The Northern blots in Fig. 4A further extend these data and illustrate that while in wild-type cells the c-*myc* mRNA was reduced to undetectable levels on days 2 and 3 of differentiation, the reduction in $IFN^r$ clone 16 was much more moderate. Hybridization of the same blots with the

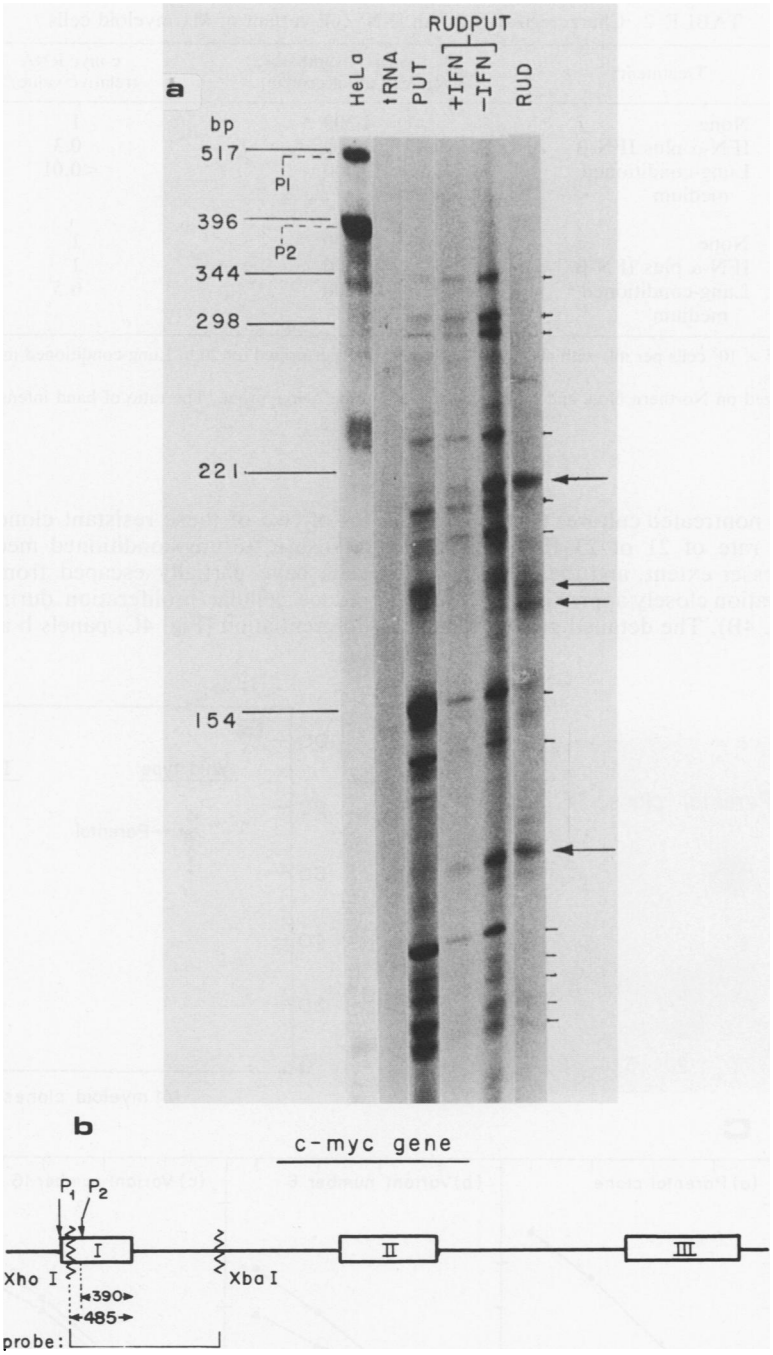


FIG. 3. RNase protection mapping of c-*myc* mRNAs synthesized in parental cells and in a somatic cell hybrid. (a) Fragments protected by poly(A)$^+$ RNA (2 μg) from PUT, RUDPUT, and RUD; in the case of RUDPUT cells, RNA was also extracted 48 h after treatment with IFN-α (200 U/ml). In addition, total RNA (20 μg) from growing HeLa cells was analyzed; the two main fragments protected in this lane are marked as P1 and P2. *Escherichia coli* tRNA (20 μg; Sigma) was used as a control. The arrows represent protected fragments in the cell hybrid originating from the RUD cells (210-, 180-, 175-, and 120-base fragments). Fragments marked by small lines originated from PUT cells. The positions of the molecular weight markers are shown at the left. (b) Strategy of RNase protection mapping; the lengths in bases of the two expected labeled RNA fragments protected by germ line c-*myc* mRNA are noted. $P_1$ and $P_2$ are the two promoters of c-*myc*, and I, II, and III are the three exons of the gene.

actin probe used as a reference clearly indicated that the β-actin mRNA levels did not change during these treatments (Fig. 4A).

The most striking difference relates to the growth behavior of the IFN$^r$ clones after exposure to the differentiation stimulus. As reported before (33) and illustrated in Fig. 4C (panel a), the growth rate of the wild-type M1 cells gradually declined following exposure to lung-conditioned medium. Screening of 13 isolated wild-type myeloid clones revealed that on day 3 of differentiation cell density was 60 to 80%

TABLE 2. Characterization of an IFN$^r$ cell variant of M1 myeloid cells

| Cells | Treatment[a] | 2',5'-oligo A synthetase (cpm/10 μg of protein) | c-*myc* RNA (relative value)[b] | Lysozyme activity (U/5 × $10^6$ cells) |
|---|---|---|---|---|
| Wild type (parental) | None | 4,300 | 1 | ND[c] |
| | IFN-α plus IFN-β | 62,000 | 0.3 | |
| | Lung-conditioned medium | 30,000 | <0.01 | 3.8 |
| IFN$^r$ (clone 16) | None | 3,300 | 1 | ND |
| | IFN-α plus IFN-β | 40,000 | 1 | |
| | Lung-conditioned medium | 25,000 | 0.5 | 3.5 |

[a] Treatment of myeloid cells (2.5 × $10^5$ cells per ml) with 400 U of IFN per ml was performed for 20 h. Lung-conditioned medium (12% [vol/vol]) was added to 1.5 × $10^5$ cells per ml for 3 days.

[b] Total RNA (20 μg) was analyzed on Northern blots and hybridized to the murine c-*myc* probe. The ratio of band intensity in treated samples to that in nontreated samples was calculated.

[c] ND, Not detected.

lower than that observed in nontreated cultures (Fig. 4B). In contrast, the proliferation rate of 21 of 23 IFN-resistant clones was inhibited to a lesser extent, and the cell density values on day 3 of differentiation closely approximated those of nontreated cultures (Fig. 4B). The detailed growth kinetics of two of these resistant clones (clones 6 and 16) after exposure to lung-conditioned medium illustrate that these cells have partially escaped from the mechanisms which reduce cellular proliferation during the first three days of differentiation (Fig. 4C, panels b and c).

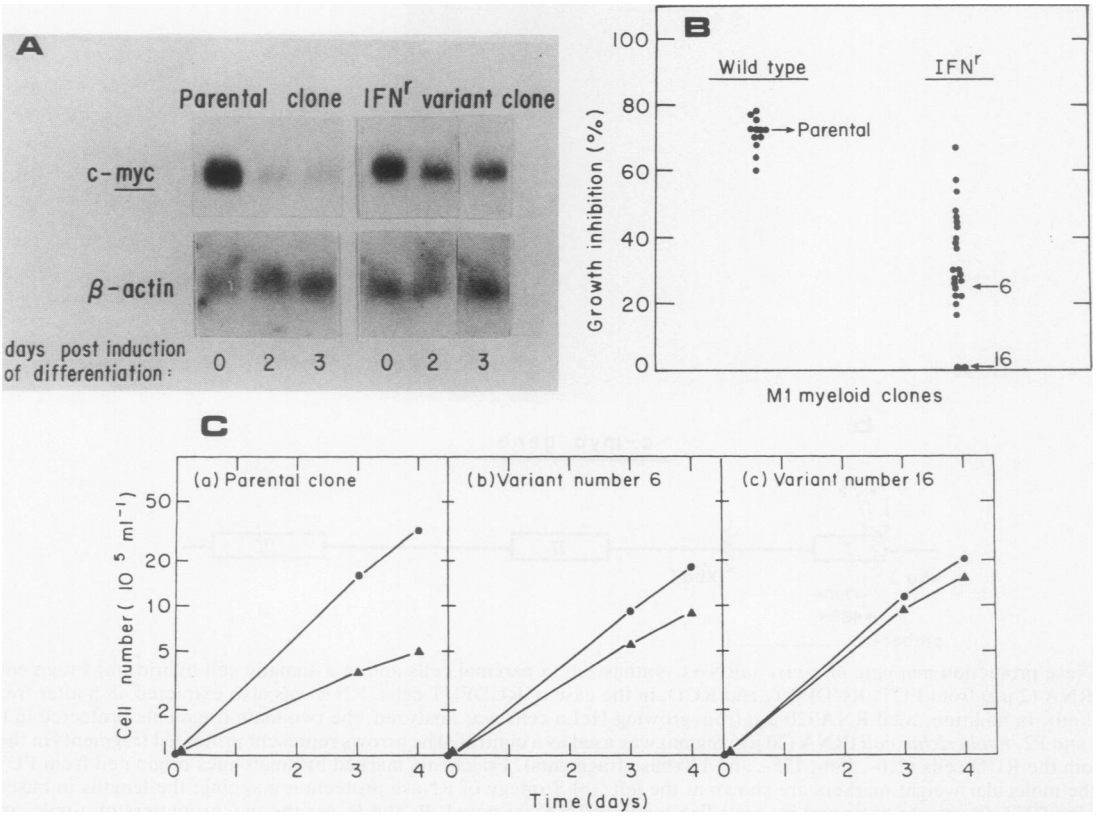


FIG. 4. Growth kinetics and c-*myc* mRNA expression during in vitro-induced differentiation of IFN$^r$ clones of M1 myeloid cells. The M1 cells were grown and induced to differentiate by lung-conditioned medium (12% [vol/vol]) as described in Materials and Methods. (A) Northern blot hybridization analysis of c-*myc* mRNA. Total RNA was extracted before and on days 2 and 3 after induction of differentiation from the parental wild-type cells and the IFN$^r$ clones. Samples (20 μg) were separated through gels, Northern blotted, and hybridized to the genomic fragment of the murine c-*myc* gene carrying the second and third exons and then to the actin cDNA probe. (B) Comparison of the extent of growth inhibition on day 3 of differentiation between IFN-sensitive and -resistant clones. The percentage of growth inhibition was calculated by using the values of cell density in control cells cultured under the same conditions as the induced cells but without the lung-conditioned medium. (C) Time course of cellular proliferation during differentiation of M1 cells. Parental cells (a) and two IFN$^r$ cell variants (b and c) were tested. Cells were counted at the indicated times after induction of differentiation. Symbols: ●, control, noninduced cells; ▲, induced cells.

## DISCUSSION

Studies of oncogene activation have so far been limited to dominant gene alterations, since the experimental approaches which were undertaken either in cell cultures (3, 16, 26) or later in transgenic mice (1, 37) were all designed to identify dominant transforming genes. Several lines of evidence suggest, however, that recessive mutations associated with the loss or inactivation of some as-yet-unidentified genes might also participate in oncogenesis. This concept is based on data showing that normal cells suppress tumorigenicity after their fusion with transformed cells (35). Chromosomal deletions which are associated with the formation of some tumors, such as retinoblastomas, Wilms' tumor of the kidney, and other embryonal tumors, also support this possibility (23). The gene whose absence or alteration apparently represents one of the recessive oncogenes that predisposes to retinoblastoma and osteosarcoma has been isolated (14, 28).

In this work we have identified a new type of genetic deregulation associated with the loss of negative control of the c-*myc* nuclear oncogene. This deregulation prevents a well-defined growth inhibitor, IFN, from suppressing c-*myc*. As a consequence, the gene is not turned-off efficiently at stages of differentiation at which it is normally suppressed by a self-secreted IFN, and cellular proliferation further proceeds. The recessive nature of this genetic deregulation in the resistant Burkitt lymphoma cell line is based on the observation that negative control of c-*myc* can be restored in stable cell hybrids. It is unlikely that the locus which confers resistance was lost during the preparation or maintenance of the hybrid cell lines, since both independently prepared cell hybrids, each containing the expected modal chromosome number, had a sensitive phenotype. Therefore, it is suggested that the loss or inactivation of elements which operate in *trans* is involved. The putative deregulated gene could code for DNA-binding proteins which interact with regulatory sequences of c-*myc* or for an upstream element in the receptor-signal transducing machinery which reduces c-*myc* expression. We propose, therefore, that in Burkitt lymphoma cells, in addition to the well-studied translocation process which leads to the dominant activation of c-*myc*, a second independent recessive defect which abrogates the down-regulation of c-*myc* by IFN also takes place in some, but not all, cases. The second genetic deregulation could represent more advanced stages in the development of the tumor. Indeed, such heterogeneity in growth responses to IFN was also detected when briefly incubated fresh biopsies from Burkitt lymphoma patients were tested. In contrast, different biopsies from the same patients were alternatively resistant or sensitive (12). Recent work with transgenic mice also suggests that in addition to the juxtaposition of c-*myc* into the vicinity of the immunoglobulin domain, an additional genetic event(s) is required to generate the fully transformed cells (1). It should be mentioned that the fusion process restored c-*myc* sensitivity but not growth sensitivity to IFN in both cell hybrids (data not shown). Therefore, the difference in the growth response to IFN between Raji and each of the two other parental cell lines involves more than one genetic change.

IFN-resistant cell variants in which the negative control of c-*myc* has been impaired can also be isolated in vitro by a direct selection procedure. We have previously selected and analyzed such a variant from Daudi Burkitt lymphoma cells (10), and in the present work we have isolated resistant clones from M1 myeloid cells. Strikingly, among all variants of Daudi and M1 cells which grew in the presence of IFN, neither cell surface receptors nor postreceptor elements which transduce some of the gene-inducing signals were inactivated. Therefore, deregulations which selectively impair the inhibitory effects of IFN on c-*myc* occur at higher frequencies than do other genetic alterations associated with responses to IFN. The exposure of IFN-resistant M1 cells to lung-conditioned medium revealed that these cells displayed an unusually high extent of c-*myc* expression and proliferative activity during the first three days of differentiation, while the production of autocrine IFN was induced normally. Thus, we provide evidence, for the first time, that release from the negative control of the self-secreted IFN leads to continuous cellular growth at stages of differentiation at which growth is usually restricted. In other words, disruption of the suppressive signals triggered by a growth inhibitor represents a new type of recessive genetic deregulation with oncogenic potential. The limited efficacy of IFN in clinical trials associated with treatment of leukemias and lymphomas could result from this type of genetic deregulation.

Finally, it should be pointed out that resistance to IFN did not completely abrogate the reduction of c-*myc* mRNA and growth during in vitro-induced differentiation and that the resistant variants of M1 myeloid cells stopped proliferating at later time points (data not shown). It is possible that other growth inhibitors besides IFN could also operate in these differentiating systems; tumor necrosis factor is currently being investigated in this context (38). We have recently reported that tumor necrosis factor reduces directly and within 1 to 3 h the transcriptional rate of c-*myc* and that it cooperates with IFN to provide maximal c-*myc* inhibition (41). Since the inhibitory effects of tumor necrosis factor and IFN on c-*myc* were found to be mediated via different molecular mechanisms (41), it is possible that resistance to IFN does not interfere with the normal response to tumor necrosis factor. A study combining two or more well-defined growth inhibitors should be performed in an attempt to explore growth suppression during terminal differentiation.

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