identified p53 abnormalities in about 20% by the RNase protection assay. In fact, we have probably underestimated the frequency of p53 abnormalities both in tumor samples and cell lines, since the RNase protection assay has a detection rate of <50% for single base pair mutations (*13, 17*).

From recent studies, including this report, it is clear that lung cancers have suffered many alterations to known or suspected anti-oncogenes. In fact, the alterations of the *Rb* and p53 genes, as well as one or more putative anti-oncogenes in chromosome region 3p, appear to coexist in ten SCLC and nine non-SCLC cell lines that we have studied for all three abnormalities (*2, 3, 6, 14*). Using cytogenetic or RFLP analysis, or both, all of the SCLCs and six of the non-SCLCs have 3p deletions or loss of heterozygosity. Probably all of the SCLC cell and at least two of the non-SCLC cell lines have abnormalities of the *Rb* gene. Thus, seven of the SCLC cell lines have all three abnormalities. Although none of the non-SCLC lines have all three abnormalities, four have a p53 and a 3p abnormality and one has a p53 and an *Rb* lesion. Future studies addressing when these lesions develop and whether their correction reverses the malignant phenotype should help us not only to understand the molecular mechanism of initiation and progression of lung cancer but provide new approaches to prevention, diagnosis, prognosis, and possibly therapy.

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Normal Expression of a Rearranged and Mutated c-*myc* Oncogene After Transfection into Fibroblasts

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Expression of the c-*myc* oncogene is deregulated in a variety of malignancies. Rearrangement and mutation of the c-*myc* locus is a characteristic feature of human Burkitt's lymphoma. Whether deregulation is solely a result of mutation of c-*myc* or whether it is influenced by the transformed B cell context has not been determined. A translocated and mutated allele of c-*myc* was stably transfected into fibroblasts. The rearranged allele was expressed indistinguishably from a normal c-*myc* gene: it had serum-regulated expression, was transcribed with normal promoter preference, and was strongly attenuated. Thus mutations by themselves are insufficient to deregulate c-*myc* transcription.

IN HUMAN BURKITT'S LYMPHOMA (BL) cells, the region of chromosome 8 that contains c-*myc* is often translocated to the immunoglobulin heavy chain (IgH) locus (*1*). The translocated allele is expressed abnormally, whereas the unrearranged copy is usually transcriptionally silent (*2*). Studies with human peripheral B cells in culture (*3*) and with transgenic mice (*4*) suggest that c-*myc* gene deregulation represents one step in the multistep generation of a fully transformed BL phenotype. All translocated c-*myc* alleles have sequence alterations upstream of exon II (*5, 6*), frequently in the vicinity of the dual promoters P1 and P2 and near the exon I–intron I boundary. In normal cells the latter region contains sites for both attenuation of ongoing transcription (manifested by greater RNA polymerase density on exon I sequences relative to intron I and exon II) and translation initiation of a large form of the c-*myc* protein (*7*). A cause and effect relationship between such mutations and deregulated c-*myc* expression has been proposed (*5, 6*); however the mechanism by which mutations might affect c-*myc* regulation has not been tested experimentally. To make such a test, a transfection system is required in which the expression patterns of both normal and mutated human c-*myc* genes can be directly compared. We now demonstrate that a BL-derived c-*myc*-IgH translocation locus that bore mutations within and proximal to the c-*myc* gene was expressed normally in transfected fibroblast cells: it was accurately transcribed from c-*myc* promoters P1 and P2 with normal promoter preference, transcription was regulated in response to serum, ongoing transcription was attenuated near the end of exon I, and the mRNA had apparently normal stability.

The c-*myc* translocation in the BL cell line AW-Ramos joins the c-*myc* and the immunoglobulin heavy chain locus within the IgM switch region (*8*). The translocation breakpoint occurs 340 bp upstream of c-*myc*

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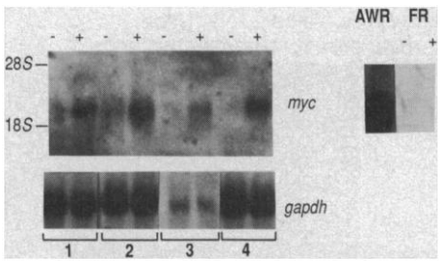

Fig. 1. Northern analysis of human c-*myc* expression in transfected fibroblast cell lines. Total cellular RNA (10 μg) isolated by the cesium chloride method (*23*) from serum-starved (−) and -stimulated (+) cells (*13*) was electrophoretically separated, transferred to nitrocellulose, and hybridized to the probes described below. Lanes 1, FR3T3 cells transfected with a normal human c-*myc* genomic fragment (*19*); lanes 2, pSV2.R transfectant r2; lanes 3, pSV2.R′ transfectant r′11; lanes 4, pSV2.R transfectant rE; AWR, total cellular RNA isolated from a growing culture of AW-Ramos BL cells; FR, FR3T3 parental cells. The c-*myc* mRNA species migrate as a broad band of 2.2 to 2.4 kb (*14*). Under the conditions used, hybridization was specific for human *myc* transcripts. Probes: nick-translated c-*myc* Cla I–Eco RI 3′ end fragment; rat *gapdh* cDNA clone (*15*).

promoter P1 (−340). The allele also has a point mutation at position −158 and at 33 bp upstream of the exon I–intron I junction (*8*). The latter mutation occurs in a region identified as a mutational hot spot in BL cells and has been suggested to play an important part in deregulating expression of this gene by increasing read-through transcription past the attenuation site (*6*). The sequence of this c-*myc* gene is otherwise normal (*8*).

We ligated the 8.4-kb fragment from an Eco RI digest of AW-Ramos DNA (the complete three-exon c-*myc* gene joined by translocation to ~1.6 kb of IgM sequence) (*8*) in both orientations into the Eco RI site of the vector pSV2.gpt. The vector contained the *Escherichia coli* guanine phosphoribosyl transferase gene (*gpt*) (expression of which in mammalian cells permits growth in the presence of mycophenolic acid) directed by the simian virus 40 (SV40) early region promoter (*9*). Recombinant vectors pSV2.R and pSV2.R′, with c-*myc* and SV40 promoters in the same and divergent transcriptional orientations, respectively, were generated. These constructs were transfected into early passage cells of the rat fibroblast cell line FR3T3 (*10*) by calcium phosphate coprecipitation (*11*). Single colonies of mycophenolic acid–resistant cells were isolated and analyzed by Southern hybridization (*12*); those with full-length human AW-Ramos c-*myc* Eco RI fragments were studied further.

To ascertain whether the growth properties of the transfected cells were altered by the presence of an "activated" allele of *myc*, we measured the DNA content of serum-starved (*13*), confluent transfectants. Flow cytometric analysis of propidium iodide–stained cells indicated a DNA content identical to that of the parental cell line (*12*).

The transfected Ramos genes express c-*myc* mRNA of the correct size [approximately 2.4 kb (*14*)] (Fig. 1). Nuclease S1 protection assays confirmed that transcription of the introduced human Ramos genes was initiated correctly at the normal c-*myc* promoters P1 and P2 (*12*). Regulation of the transfected Ramos gene was normal: expression increased after addition of fetal calf serum (15%) to starved cell cultures (*13*) (Fig. 1). The serum response was specific, as compared to the constitutive expression of the rat glyceraldehyde phosphate dehydrogenase gene (*gapdh*) (*15*) (Fig. 1). Serum induction was confirmed by ribonuclease protection analysis of human and endogenous rat c-*myc* RNA from serum-starved and -stimulated cells (Fig. 2). Serum-stimulated expression from both promoters P1 and P2 of the human gene. Although P1-initiated transcripts were detectable [as in (*16*)], the P2 promoter was preferred, in contrast to the behavior of the translocated gene in the AW-Ramos BL cell line, in which P1 was utilized more (Fig. 2A, lane 10). The kinetics of transfected gene stimulation by serum were consistent with mitogen stimulation of endogenous c-*myc* (*17*): the increase in mRNA peaked about 1 hour after serum addition, and declined by 100 min (Fig. 2A). Expression of the human Ramos c-*myc* gene in the transfected cells was comparable to the endogenous genes, although rat P1-derived mRNA was not detected (Fig. 2B). The endogenous genes were expressed despite the presence of a translocated c-*myc* allele, in contrast to unrearranged c-*myc* genes in BL cells (*2*). Altogether, seven cell lines transfected with pSV2.R or pSV2.R′ were assayed by either nuclease S1 or ribonuclease protection, and all exhibited reproducible serum-responsive c-*myc* expression.

Mutation of exon I–intron I junction sequences has been proposed as a general mechanism of deregulating c-*myc* expression in BL cells, by reducing or eliminating the transcription block that normally occurs at the end of exon I (*6*). Typical of almost all BL cells examined, AW-Ramos cells exhibit similar densities of RNA polymerase on exon I, intron I, and exon II c-*myc* sequences (*6*) (Fig. 3A). The extent to which transcrip-

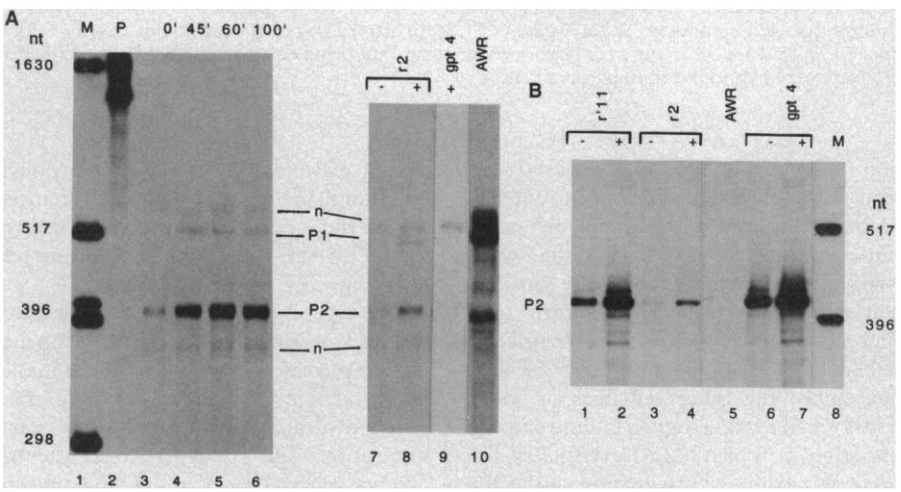

Fig. 2. Ribonuclease protection analysis of serum-regulated c-*myc* expression. Assays were done with ribonucleases A (Sigma) and T1 (Boehringer Mannheim). Reaction products were analyzed on 6% polyacrylamide sequencing gels. Identical results were obtained from at least two independent experiments. M, molecular weight markers prepared by Hinf I digestion of pAT153 plasmid DNA labeled with Klenow (fragment sizes in nucleotides); P, unreacted probe; P1 and P2, specific protection of transcripts derived from *myc* promoters; n, nonspecific protection products. (**A**) Analysis of transfected human c-*myc* transcripts (5 μg of total RNA per lane). Probe: 876-nucleotide (nt) antisense RNA probe complementary to human c-*myc* exon I sequences prepared by T7 polymerase (Promega) transcription of an Xho I–Sac I fragment cloned in the Bluescript vector (Stratagene). This probe spans most of exon I, distinguishes P1 from P2, and is specific for human c-*myc*; note absence of specific protection in control lane 9 (pSV2.*gpt* transfectant gpt 4). Time course of human c-*myc* serum response in cell line r′11 (lanes 3 to 6). Total cellular RNA was isolated from starved cells at the indicated times after stimulation with 15% FCS. Serum induction of r2 (lanes 7 and 8). RNA from serum-deprived (−) and -stimulated (+) cell cultures 60 min post serum addition (*13*). Lane 10, RNA from the BL cell line AW-Ramos. (**B**) Expression of endogenous rat c-*myc* genes (10 μg of total RNA analyzed). Probe: T7 polymerase antisense transcript of fragment spanning rat c-*myc* exon I, cloned in pGem4 (Promega Biotek). This probe is specific for rat *myc* (note the absence of protection in lane 5, AW-Ramos RNA). Lanes 6 to 7, expression of rat c-*myc* genes in transfectants r′11 and r2 in response to serum deprivation and stimulation. Lanes 6 and 7, cell line gpt 4.

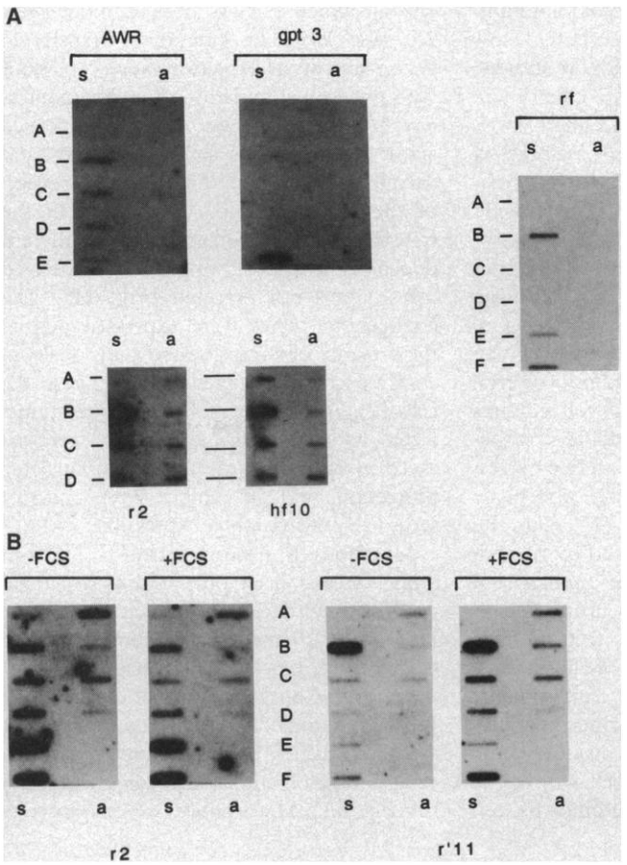

Fig. 3. Transcriptional activity of human c-*myc* genes in nuclear run-on assays. Nuclei were prepared from AW-Ramos tumor cells and density-arrested fibroblasts. Run-on transcription assays were done essentially as described (*24*). Preparation of filters, hybridization, and washing were as described (*19*). Single-stranded M13 probes from the human c-*myc* gene: A, 535-bp Sma I fragment detects transcription initiating ~600 nucleotides upstream of exon I (*25*); B, 443-bp Xho I–Pvu II exon I fragment; C, 606-bp Sst I fragment from intron I; D, 414-bp Pst I fragment from exon II. Both sense (s) and antisense (a) c-*myc* probes were used. Probes E and F are *gapdh* and pSV2.*gpt* plasmid DNA clones, respectively. (**A**) Transcription in AW-Ramos tumor cells (AWR), control fibroblast line gpt 3 (transfected with vector pSV2.*gpt*), pSV2.R transfected fibroblast lines r2 and rf, and fibroblast line hf10 transfected with a normal genomic human c-*myc* construct (*19*). (**B**) Transcriptional serum response in fibroblast cell lines r2 and r'11. Nuclei were prepared from density-arrested cell cultures that were serum-starved for 4 days (−FCS), or starved and then treated with 15% FCS for 1 hour (+FCS). Run-on assays were performed simultaneously, and equal amounts of labeled reaction products were hybridized to duplicate filters.

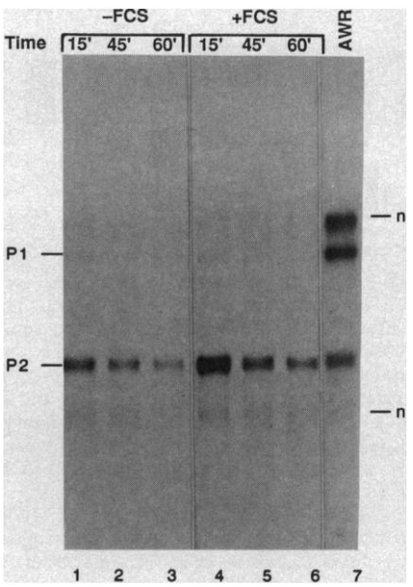

Fig. 4. Stability of human Ramos c-*myc* mRNA in pSV2.R′ transfectant r′11. Total cellular RNA (5 μg per lane) analyzed by ribonuclease protection. The probe is described in Fig. 2A. Lanes 1 to 3, density-arrested serum-starved cells (−FCS) were treated with actinomycin D (Act. D, 5 μg/ml; Boehringer Mannheim). RNA was isolated at the indicated times after Act. D treatment. Lanes 4 to 6, serum-starved cultures were stimulated with 15% serum (+FCS) for 45 min, Act. D (5 μg/ml) was then added and RNA isolated at the indicated times after addition of the inhibitor. Lane 7, RNA from the BL cell line AW-Ramos. P1 and P2, specific protection of human c-*myc* transcripts; n, nonspecific protection.

tion of Ramos-derived c-*myc* genes was attenuated in transfected growth-arrested fibroblast cultures was determined by nuclear run-on assays (*18*). In these cells, the Ramos c-*myc* gene was attenuated to the same extent as a transfected normal human c-*myc* genomic clone (Fig. 3A). The transfected cell line rf bears an intact Ramos c-*myc* gene but, unlike the other cell lines, it had a transformed morphology in culture and was tumorigenic in vivo (*12*). Nevertheless, this clone also exhibited attenuation of the Ramos allele (Fig. 3A). Thus, relief of attenuation in BL cells is not a straightforward consequence of the transformed phenotype itself.

The rapid serum induction of c-*myc* is an important regulatory mechanism which was not altered by proximal mutations associated with BL-derived c-*myc* alleles. Normal human c-*myc* genes are not stimulated transcriptionally by the addition of serum in transfected FR3T3 cells, as assayed in nuclear run-on experiments (*19*). Similarly, in cells transfected with the Ramos-derived allele of c-*myc*, exon I signal strength was unaffected by addition of serum (Fig. 3B). The linked SV40 promoter on the transfecting plasmid, however, was reproducibly stimulated by serum, which demonstrated the independent transcriptional activities of the c-*myc* and viral promoters (*19*) (Fig. 3B).

This experiment also indicated that mitogen responsiveness of the transfected genes may be mediated in part through the relief of attenuation, manifested as more transcription of intron I sequences after serum stimulation. The extent to which attenuation was relieved varied among the different transfected lines (for example, compare r2 and r′11 in Fig. 3B); therefore this mechanism accounts for only some of the observed serum response.

Point mutation of exon I could modify post-transcriptional regulation of RNA abundance, thereby contributing to deregulated c-*myc* expression. RNA transcribed from a normal transfected human c-*myc* gene has a half-life of 20 to 30 min in both serum-starved and -stimulated cell cultures (*19*), consistent with previous reports for normal c-*myc* transcripts (*20*). Messenger RNA transcribed from the transfected Ramos c-*myc* allele was similarly unstable (Fig. 4).

Our results indicate that a rearranged and point-mutated c-*myc* allele cloned from a BL cell line is capable of apparently normal, regulated expression when transfected into fibroblast cells. Such an allele of c-*myc* cannot, therefore, be considered an obviously deregulated gene outside of the context of a transformed B cell environment. In BL, deregulated c-*myc* expression may be the result of alterations in trans-acting mechanisms operative in specific B cells. Alternatively, juxtaposition of c-*myc* and Ig locus sequences may cause abnormal usage of the P1 promoter (*21*), and these transcripts may not be subject to, for example, strict attenuation control. The introduction of rearranged c-*myc* genes into BL cells by transfection might, in the future, aid in distinguishing between these possibilities. However, normal c-*myc* genes are not transcribed in BL cells, and preliminary data (*12*) indicate that transfected human c-*myc* genes are similarly unexpressed, possibly through the activity of a tumor cell–specific repressor factor (*22*). Nonetheless, the model c-*myc* translocation locus studied here requires a particular environment in order to express the deregulated transcription pattern characteristic of BL-associated c-*myc* genes.

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Mutants of Pertussis Toxin Suitable for Vaccine Development

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Immunization with chemically detoxified pertussis toxin can prevent severe whooping cough with an efficacy similar to that of the cellular pertussis vaccine, which normally gives unwanted side effects. To avoid the reversion to toxicity and the loss of immunogenicity that may follow chemical treatment of pertussis toxin, inactive toxins were constructed by genetic manipulation. A number of genetically engineered alleles of the pertussis toxin genes, constructed by replacing either one or two key amino acids within the enzymatically active S1 subunit, were introduced into the chromosome of strains of *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica*. These strains produce mutant pertussis toxin molecules that are nontoxic and immunogenic and that protect mice from the intracerebral challenge with virulent *Bordetella pertussis*. Such molecules are ideal for the development of new and safer vaccines against whooping cough.

WHOOPING COUGH, AN ACUTE respiratory disease affecting over 60 million infants and responsible for approximately 1 million deaths each year, can be prevented by vaccination (*1*). The vaccine currently used, containing killed *Bordetella pertussis* cells (*2*), although effective, has been associated with rare neurological complications and deaths (*3*), which have decreased the acceptance of the vaccine in many countries (*4*). As a result, pressure has built up to develop safer, acellular vaccines against whooping cough (*4*), and a number of vaccines have been proposed.

Pertussis toxin (PTX), a major virulence factor of *Bordetella pertussis*, either alone or combined with other antigens, is the main component of all acellular vaccines so far developed (*5*). A large-scale clinical trial carried out in Sweden has shown that chemically detoxified PTX can prevent severe whooping cough with an efficacy equal to or close to that of the cellular vaccine (*6*). Unfortunately, the chemical methods used to detoxify PTX are not completely satisfactory, since they give a product with reduced immunogenicity, which in some cases has been shown to revert to toxicity (*7*). Complete and stable detoxification of PTX is mandatory, since the severe complications of the cellular vaccine that can lead to permanent neurological damage and death may be due to minute traces of residual toxin activity (*8*). The approach that we have adopted is aimed at genetic detoxification.

Pertussis toxin is a complex bacterial protein toxin composed of five noncovalently linked subunits called, according to their

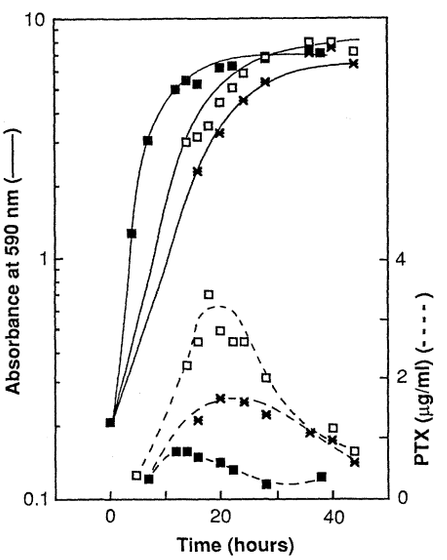

Fig. 1. Production of PTX-129G by *B. pertussis* W28 (X), *B. parapertussis* P14 (□), and *B. bronchiseptica* 7865 (■). Bacteria were grown in 125-ml flasks containing 15 ml of Steiner-Scholte medium at 35°C for 72 hours. The amount of PTX present in the supernatant was determined by enzyme-linked immunoassay (*11*). Standard deviation for each data point was less than 15%. The other PTX mutants listed in Table 1 were also produced by the three *Bordetella* species with a similar pattern.

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