Augmented Expression of Normal c-myc Is Sufficient for Cotransformation of Rat Embryo Cells with a Mutant ras Gene

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We studied the effect of altered c-myc structure and expression upon the ability of c-myc to promote the transformation of normal rat embryo cells when it was supplemented by EJras (the mutant c-H-ras1 gene from EJ/T24 bladder carcinoma cells). We tested several c-myc alleles cloned from normal and tumor tissues of chicken and human origin and found that only LL4myc (derived from a bursal lymphoma in which an avian leukosis virus long terminal repeat resides within the first c-myc intron in the same transcriptional orientation) had cotransforming activity. No activity was observed with normal chicken and human c-myc alleles, two other bursal lymphoma c-myc alleles (LL3myc and LL6myc), and two human c-myc genes (HSRmyc and DMmyc) from human neuroectodermal tumor cell line COLO320, in which c-myc is amplified. Some of these inactive alleles had the following alterations that are frequently found in tumor-derived c-myc: point mutations affecting the encoded protein (LL3myc); a truncated structure with loss of the first, noncoding exon (LL3myc and DMmyc); and proviral integration within or near the myc locus (LL3myc and LL6myc). The following two experimental approaches indicated that cotransforming activity was directly related to the transcriptional activity of the alleles in cultured rat cells: (i) when cotransfected into Rat-2 cells, LL4myc was more highly expressed than the other (inactive) alleles; and (ii) augmented expression of HSRmyc, DMmyc, or normal human or normal chicken c-myc placed under the transcriptional control of retroviral long terminal repeats or increased expression of normal human c-myc under the influence of a retroviral enhancer element was accompanied by cotransformation activity. We concluded that augmented expression of even a normal c-myc gene is sufficient for cotransforming activity and that additional structural alterations frequently found in tumor-derived alleles are neither necessary nor sufficient for the gene to acquire rat embryo cell cotransforming properties.

The cellular *myc* gene (c-*myc*) has been implicated in the development of tumors in many species of animals. Initially identified as the cellular homolog of the viral *myc* oncogene (v-*myc*) present in several avian retroviral isolates which induce a broad range of malignancies (17, 42, 51), the c-*myc* locus is altered and presumably pathogenic in many tumors (for a review, see reference 49). These alterations include integration of retroviral elements within or near the c-*myc* locus in lymphomas of birds (23, 36), rodents (9, 46), and cats (34); gene amplification in various types of tumors (3, 7, 12, 29); and chromosomal translocations and breakpoints within or adjacent to c-*myc* in human Burkitt's lymphoma (1, 11, 48), mouse plasmacytomas (1, 10, 32, 43, 48), and rat immunocytomas (47).

The normal human (20, 52, 53), mouse (45), and chicken (44) c-*myc* genes include three exons which give rise to a mature mRNA that is approximately 2.1 kilobases (kb) long (53). Sequences from exon 1 become part of a long, untranslated leader (20, 44, 45, 52, 53), whereas sequences from exons 2 and 3 code for the c-*myc* protein, a nuclear phosphoprotein approximately 430 amino acids long (2, 21, 22, 39). Tumor-associated alterations of c-*myc* frequently truncate the gene; e.g., insertion of retroviral proviruses and chromosomal translocation often separate the coding exons from the first exon and the normal regulatory signals (44, 45). In addition, nucleotide substitutions and deletions that alter the encoded protein (38, 54) or putative regulatory domains (38) have been described. The many observed mutations and rearrangements of c-*myc* are presumed to provide a selective advantage for tumor cell growth in vivo or in culture by supplying an oncogenic function. This function may be influenced by augmented expression (as occurs upon gene amplification or insertion of a retroviral provirus), by more subtle disturbances of gene regulation (as proposed to occur after translocation), by alterations in the structure of the c-*myc* protein, or by combinations of these changes.

A major obstacle to studying the functional roles of tumor-derived, mutant c-*myc* alleles in oncogenesis has been the scarcity of convenient assays for the phenotypic consequences of c-*myc* alterations. Unlike the active cellular *ras* oncogenes, which efficiently induce focus formation in established rodent fibroblast cell lines, c-*myc* genes cloned from tumors produce no obvious morphologic alterations and few growth alterations in these recipient cells (8, 25). However, the discovery that the mutant *ras* gene from bladder carcinoma cell line EJ/T24 (EJras) (35) induces focus formation in normal rat embryo cells (REC) only in conjunction with other genes, including a tumor-derived *myc* gene whose expression is augmented by the simian virus 40 early promoter (28), provided an assay for the oncogenic activity of c-*myc* alleles. Accordingly, we used the REC cotransformation assay to determine whether various normal c-*myc* genes, altered c-*myc* alleles cloned directly from tumor cells, and c-*myc* clones designed in vitro can assist transformation of normal REC by EJras. Our results indicate that the cotransforming activity of c-*myc* requires only alterations that augment c-*myc* expression.
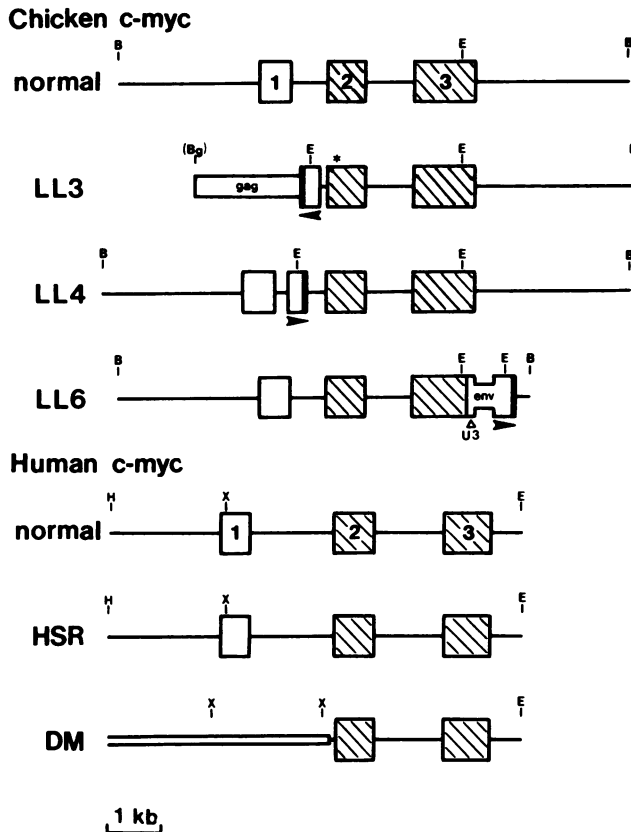
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Chicken c-myc

FIG. 1. c-myc clones from normal and tumor cells of chicken and human origin used in cotransformation experiments. The alleles of chicken origin included the normal gene and three alleles from ALV-induced bursal lymphomas (LL3myc, LL4myc, and LL6myc). The human alleles included the normal gene and two alleles isolated from different sublines of COLO320 cells; HSRmyc was cloned from the subline containing marker chromosomes with homogeneously staining regions, and DMmyc was cloned from the subline with double minutes. The three c-myc exons are indicated for the normal genes, and the coding regions are cross-hatched. The retroviral elements present in the c-myc loci cloned from bursal lymphomas are indicated by arrowheads beneath the LTRs showing their transcriptional orientation; the U3 portions of the LTRs are indicated by open boxes, and the R and U5 portions are indicated by solid boxes. Retroviral structural genes that are present in the clones are labeled. An asterisk indicates the position of a base pair substitution in LL3myc which results in an altered amino acid composition of the encoded protein. The DMmyc locus is truncated, and the coding exons are adjacent to a relocated segment of DNA (open narrow box). Restriction sites for the following enzymes are indicated: B, BamHI; E, EcoRI; (Bg), former BglII site; H, HindIII; and X, XhoI. Additional details are provided in Materials and Methods.

MATERIALS AND METHODS

c-myc clones and recombinants. The normal chicken c-myc gene [c-myc(ch)] is contained within a 10-kb BamHI-BamHI fragment cloned in pBR328 (51) (Fig. 1). LL3myc, LL4myc, and LL6myc are pBR322 clones of chicken bursal lymphoma c-myc alleles (36). Clone LL3myc extends from the BglII site in the avian leukosis virus (ALV) gag gene to a BamHI site 3' to the c-myc exons and contains only the 5' portion of the provirus integrated into the first c-myc intron; the LL3 provirus has incurred a 1.1-kb deletion of the 5' gag region, but contains an intact 5' long terminal repeat (LTR). The

c-myc coding domain of LL3myc contains point mutations, at least one of which leads to an amino acid substitution in the encoded protein (54). LL4myc is a BamHI-BamHI clone with a single intact ALV LTR inserted in the first c-myc intron in the same transcriptional orientation as the c-myc exons. LL6myc is a BamHI-BamHI clone containing a highly deleted provirus inserted into the 3' end of c-myc and in the same transcriptional orientation; integration of the LL6 provirus has removed the downstream polyadenylation signal of exon 3 of LL6myc, leading to an abnormally long transcript which terminates in the provirus (C. Nottenburg, personal communication). This provirus has suffered a large deletion extending from U3 in the 5' LTR to a region within env. The 3' BamHI site is from an as-yet-unidentified region of the chicken genome (36; Nottenburg, personal communication). The nucleotide sequences of the coding exons in LL4myc and LL6myc have not been determined.

Normal human c-myc [c-myc(hu)] is a HindIII-EcoRI clone in a pUC vector (a gift from W. Colby and A. Levinson, Genentech, South San Francisco, Calif.). HSRmyc and DMmyc are clones of c-myc alleles that are present in human neuroectodermal tumor cell line COLO320 (3). HSRmyc is an allele that was isolated from a tumor cell subline possessing marker chromosomes with homogeneously staining regions containing amplified copies of c-myc; DMmyc is an allele that was isolated from a subline possessing double minute chromosomes. HSRmyc has a three-exon structure indistinguishable by restriction enzyme analysis from the structure of c-myc(hu), whereas DMmyc has a truncated, two-exon structure which has been joined to DNA of uncertain origin at the 3' end of the first intron (M.S., unpublished data). Both HSRmyc and DMmyc were used as λ clones, and HSRmyc was also used as a HindIII-EcoRI subclone in pBR322.

Recombinant c-myc clones in which the addition of proviral promoter-enhancer elements activated transcription (Fig. 2) were derived from the c-myc clones described above. ΔLL4myc was derived from LL4myc by deleting sequences upstream from the SmaI site in the first c-myc intron and 5' to the LL4 LTR. LL4 LTR-c-myc(ch) was constructed by replacing the segment of LL4myc from the SmaI site between the LL4 LTR and LL4myc exon 2 to the BamHI site downstream from LL4myc exon 3 with the equivalent fragment from c-myc(ch). Murine leukemia virus (MLV) LTR-HSRmyc and MLV LTR-c-myc(hu) were made by replacing the HindIII-XhoI fragment of HSRmyc and c-myc(hu) with the HindIII-SalI fragment from pMP-1, a clone which contains the Moloney MLV (Mo-MLV) LTR from a HindIII site in the 5' flanking cellular DNA sequence to the R portion of the LTR, where a polylinker containing a SalI site was previously joined at the KpnI site (a gift from M. Scott). MLV LTR-DMmyc was constructed by ligating the 3.8-kb XhoI-EcoRI fragment of DMmyc, which contains the two c-myc coding exons, to the HindIII-XhoI fragment of pMX070, a clone which contains Mo-MLV sequences from a HindIII site in the 5' flanking cellular DNA sequence to the XhoI site of gag (a gift from M. Scott).

c-myc(hu) recombinants containing the Mo-MLV enhancer sequence (24) (Fig. 3) were obtained by inserting the HindIII-SacI fragment of pMP-1 into various sites around the normal human c-myc locus. The ends of this enhancer fragment were converted to HindIII or EcoRI ends and inserted in either orientation into the HindIII site 5' or the EcoRI site 3' to the c-myc exons [Fig. 3, Enh-c-myc(hu) 1a, 1b, 3a, and 3b]. Introduction of the MLV enhancer fragment into the PvuII site 0.4 kb upstream from c-myc exon 1 was

accomplished by converting the *Pvu*II site to a *Sac*I site and replacing the upstream c-*myc* flanking sequence with the *Hind*III-*Sac*I fragment from pMP-1 [Fig. 3, Enh-c-*myc*(hu) 2a]. A c-*myc* gene enhanced by a more highly deleted MLV LTR was derived from Enh-c-*myc*(hu) 2a by removing the *Xba*I-*Sac*I fragment of the LTR [Fig. 3, Enh-c-*myc*(hu) 2b]. To put the enhancer fragment in an orientation opposite to c-*myc*, the *Cla*I site in the 5' flanking cellular sequence of the MLV LTR was converted to a *Sac*I site, and the *Sac*I-*Sac*I fragment containing the MLV enhancer element was reversed in its orientation [Fig. 3, Enh-c-*myc*(hu) 2c].

MC29 v-*myc* is present in plasmid pMC38 (50). EJ*ras* is the 6.6-kb *Bam*HI-*Bam*HI clone present in pEJ6.6 (35) (a gift from R. Weinberg, Massachusetts Institute of Technology). Chicken B-*lym* is present in plasmid pHλ (16) (a gift from G. Cooper, Dana Farber Cancer Institute). A eucaryotic expression plasmid containing the herpes simplex virus thymidine kinase gene (pMV103) was a gift from C. Lau.

Transfection of cells. REC were transfected by using the procedure of Land et al. (28). Fourteen-day-old Fisher rat embryos were dispersed into a single-cell suspension by mechanical disruption and trypsin treatment. This suspen-
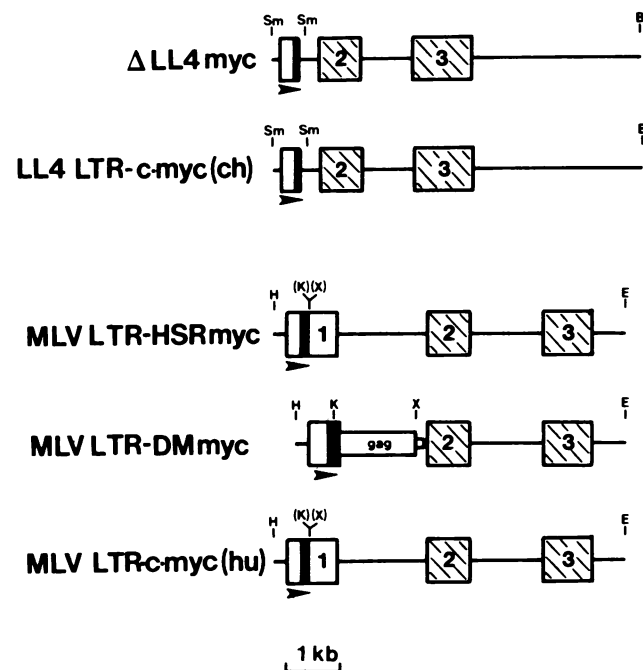

FIG. 2. Recombinant c-*myc* clones that were transcriptionally activated by retroviral LTRs. ΔLL4*myc* is a truncated version of LL4*myc* which has had the 5' portion removed up to the *Sma*I site upstream from the LL4 (ALV) LTR. LL4 LTR-c-*myc*(ch) is similar to ΔLL4*myc*, but the c-*myc* coding domain (*Sma*I-*Bam*HI fragment) of normal chicken c-*myc* has been substituted for the equivalent domain of LL4*myc*. MLV LTR-HSR*myc* and MLV LTR-DM*myc* have the Mo-MuLV LTR controlling the transcription of HSR*myc* and DM*myc*, respectively. In MLV LTR-HSR*myc*, U3 and R of the LTR are used, whereas in MLV LTR-DM*myc*, the complete LTR and almost 1 kb of *gag* are used. MLV LTR-c-*myc*(hu) is a construction similar to MLV LTR-HSR*myc* in which the c-*myc* portion of HSR*myc* has been substituted with the equivalent domain of normal human *myc*. Exons of c-*myc* are indicated, and coding exons are cross-hatched. Restriction sites for the following enzymes are indicated: Sm, *Sma*I; B, *Bam*HI; H, *Hind*III; K, *Kpn*I; X, *Xho*I; and E, *Eco*RI.
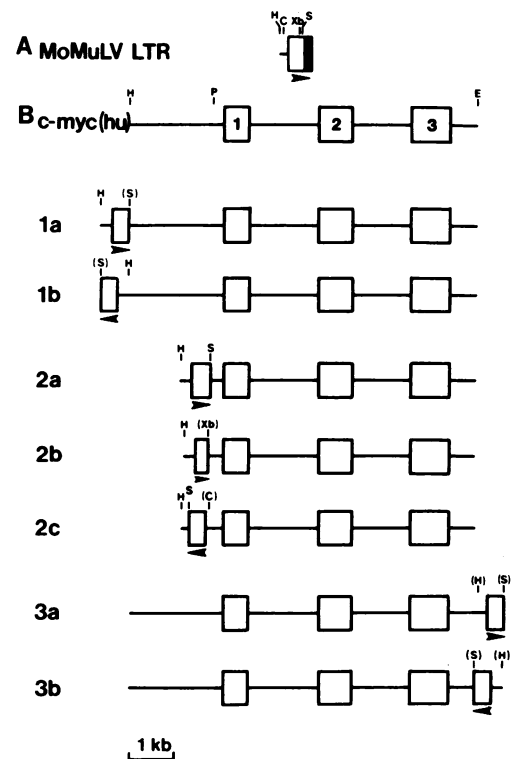

FIG. 3. Normal human c-*myc* clones that contain the enhancer portion of the Mo-MuLV LTR. (A) Entire Mo-MuLV LTR from which the enhancer fragment was obtained and a few relevant restriction enzyme sites. The solid box represents R and U5, and the open box represents U3. (B) Normal human c-*myc* locus from the *Hind*III site to the *Eco*RI site. (1a and 1b) Recombinant c-*myc* clones with insertion of the *Hind*III-*Sac*I enhancer fragment into the *Hind*III site. (2a, 2b, and 2c) Recombinant c-*myc* clones in which the enhancer fragment was substituted for the *Hind*III-*Pvu*II segment that flanked the 5' end of c-*myc*. (3a and 3b) Clones with insertion of the *Hind*III-*Sac*I enhancer fragment into the *Eco*RI site. The arrowheads indicate the orientations of the LTR fragments used. Restriction enzyme abbreviations: H, *Hind*III; P, *Pvu*II; E, *Eco*RI; C, *Cla*I; S, *Sac*I; Xb, *Xba*I.

sion was plated onto 100-cm² dishes at a density of one embryo per plate, and the preparations were cultured in Dulbecco minimal essential medium supplemented with 10% fetal calf serum, penicillin, and streptomycin under 5% CO_2. After 3 to 4 days, the cells (approximately 10^7 cells per plate) were split 1:5 and allowed to grow overnight. On the next day the medium was replaced with 5 ml of fresh medium, and 1.25 ml of calcium phosphate-precipitated DNA in 1× HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid)-buffered saline (18) was distributed over the cells. The precipitated DNA contained 6 μg of pEJ6.6 and an equimolar amount of a *myc*-containing plasmid or bacteriophage. After 5 h, the medium was replaced with 10 ml of fresh medium. On the next day, the cells were split 1:3, and the medium was subsequently changed at 3- to 4-day intervals. Foci began to appear after 7 days and reached a maximum after 14 to 21 days. To minimize the contribution of secondary foci, the foci were usually counted 14 days after transfection. The cells in foci were recovered with the aid of a cloning cylinder.

The procedure used for transfection of Rat-2 cells (tk⁻) was similar to the procedure used for REC, except that 0.1 to 1.0 μg of a plasmid containing the herpes simplex virus

thymidine kinase gene was used with a 10-fold molar excess of a nonselectable plasmid. The cells were split 1:5 on the day after transfection, and hypoxanthine-aminopterin-thymidine selection of transfected cells was applied 1 day later.

Isolation of DNA and RNA. Extraction of genomic DNA and poly(A)$^+$ RNA from cells was performed as previously described (41). Total cellular RNA was extracted by lysis with sodium lauryl sarkosinate in a solution containing 4 M guanidinium isothiocyanate and β-mercaptoethanol and was purified by ultracentrifugation through cesium chloride (6, 15).

Analysis of DNA and RNA. The procedures used for electrophoresis of nucleic acids, transfer to nitrocellulose filters, and annealing with radiolabeled probes have been described previously (41). Human c-*myc* transcripts were also analyzed by protection from RNase digestion of radiolabeled RNA complementary to human *myc* exon 1 mRNA. This probe was generated by transcription from an SP6 promoter with SP6 RNA polymerase (19). A 10-μg portion of total cellular RNA was hybridized to 10^6 cpm of this probe in 10 μl of a solution containing 75% formamide, 0.1% sodium dodecyl sulfate, 400 mM NaCl, 20 mM Tris, and 1 mM EDTA (pH 7.0) at 52°C for 12 to 16 h. RNase T$_1$ (4 μg/ml; 7,000 U/ml) and RNase A (8 μg/ml; Boehringer Mannheim Biochemicals) in 150 μl of 300 mM NaCl–10 mM Tris–5 mM EDTA (pH 7.5) were added and allowed to digest the preparation for 1 h at 25°C, after which 10 μl of 10% sodium dodecyl sulfate and 5 μl of proteinase K (10 mg/ml) were added for 10 min at 37°C. The RNA was precipitated by adding 400 μl ethanol, chilling the preparation in an ethanol-dry ice bath for 20 min, and centrifugation. The precipitate was washed with 70% ethanol and dried, after which it was dissolved in 10 to 15 μl of formamide loading buffer (95% formamide, 10 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol blue). The samples were electrophoresed on a 4% polyacrylamide–7 M urea gel by using Tris-boric acid-EDTA buffer, and the gel was exposed to Kodak XAR-5 X-ray film at −70°C.

Radiolabeled probes. DNA probes were radiolabeled with [α-^{32}P]dCTP or [α-^{32}P]dATP (Amersham Corp.) or both by nick translation (31) of gel-purified fragments. The following fragments were used: MC29 v-*myc* (*Pst*I fragment from pMC38) (50) for detection of chicken c-*myc* and MC29 v-*myc*; human c-*myc* exon 2 [*Pst*I fragment from c-*myc*(hu)] for human c-*myc*; and v-Ha-*ras* (*Eco*RI fragment of BS9) (14) for EJ*ras*. For RNase protection studies, the *Xho*I-*Pvu*II fragment of normal human c-*myc* exon 1 was subcloned into the SP65 vector, and the DNA cleaved 3' to the insertion site was used to direct synthesis of radiolabeled RNA complementary to human c-*myc* exon 1 mRNA by SP6 RNA polymerase (New England Nuclear Corp.). [α-^{32}P]UTP (Amersham) was the radioactive ribonucleotide triphosphate used for making the RNA probe. Full-length RNA was purified by electrophoresis through a 4% polyacrylamide–7 M urea gel prior to use.

RESULTS

Cotransformation of REC by c-*myc* from normal and tumor tissues. We first determined whether c-*myc* alleles cloned directly from tumor cells could induce focus formation in REC in conjunction with EJ*ras*. The cloned alleles used were isolated from three chicken bursal lymphomas and from human tumor cell lines. The three c-*myc* alleles cloned from ALV-induced bursal lymphoma cells (Fig. 1) included LL4-*myc*, which has an ALV LTR inserted in the first c-*myc*

intron in the same transcriptional orientation (36); LL3*myc*, which has part of an ALV provirus inserted in the first c-*myc* intron, but oriented opposite to c-*myc* (36, 54); and LL6*myc*, which has a severely deleted ALV provirus inserted downstream from the c-*myc* coding sequence in the same transcriptional orientation (36) (see Materials and Methods) (Fig. 1). In the case of LL4*myc*, the arrangement is such that transcription of the c-*myc* allele can be promoted by the viral LTR, whereas in the case of both LL3*myc* and LL6*myc*, the LTR may enhance but cannot promote c-*myc* expression. Limited nucleotide sequencing of LL3*myc* has revealed nucleotide substitutions, one of which is predicted to alter the encoded c-*myc* protein (54). The RNA from the LL6*myc* allele in the original tumor is polyadenylated at a site in the downstream LTR, resulting in an abnormally long mRNA which contains *env* and U3 sequences (Nottenburg, personal communication).

Two human tumor c-*myc* alleles were used; these alleles were derived from sublines of neuroectodermal tumor cell line COLO320 which contained homogeneously staining regions or double minute chromosomes and 20- to 40-fold amplifications of c-*myc* (2). HSR*myc* has three exons and a structure which is indistinguishable from that of the normal human gene by restriction enzyme analysis (M.S., unpublished data). DM*myc* has only the two coding exons, with a relocated segment of DNA joined to the c-*myc* gene just 5' to the second exon. Normal chicken c-*myc* and human c-*myc*

TABLE 1. Cotransforming activities of c-*myc* alleles and recombinants

Allele or recombinant	Focus formation	Efficiency[a]	Tumorigenicity[b]
v-*myc* (MC29)	+	25-100	NT[c]
c-*myc*(ch)	−		−
LL3*myc*	−		−
LL4*myc*	+	25-100	+
LL6*myc*	−		−
c-*myc*(hu)	−		−
HSR*myc*[d]	−		−
DM*myc*[e]	−		−
LL4*myc*	+	25-100	NT
LL4 LTR-c-*myc*(ch)	+	25-100	NT
MLV LTR-HSR*myc*	+	50-500	+
MLV LTR-DM*myc*	+	50-500	+
MLV LTR-c-*myc*(hu)	+	50-500	+
Enh-c-*myc*(hu) 1a	+	25-250	NT
Enh-c-*myc*(hu) 1b	+	25-250	NT
Enh-c-*myc*(hu) 2a	+	25-250	NT
Enh-c-*myc*(hu) 2b	+	25-250	NT
Enh-c-*myc*(hu) 2c	+	25-250	NT
Enh-c-*myc*(hu) 3a	+	25-250	NT
Enh-c-*myc*(hu) 3b	+	25-250	NT

[a] The number of foci formed after 12 to 21 days following transfection of 5 × 10^5 to 10^6 REC with 6 μg of pEJ6.6 (10.8 kb) and an equimolar amount of cloned c-*myc*. The efficiency values are the results of two or more transfection experiments.

[b] Cells (10^4 to 10^6) were tested in 4- to 6-week-old nude mice by subcutaneous injection after 200 rads of total body irradiation (28). Tests were performed with cells from confluent 100-cm^2 plates (about 2 × 10^6 cells) transfected 2 to 4 weeks earlier with the indicated genes or with 10^4 to 10^6 cloned cells originally picked as foci of transformed cells. Tumor formation was monitored over a 6 to 8-week period. Cells transfected with each of the tested combinations were either tumorigenic (+) or nontumorigenic (−) in multiple experiments.

[c] NT, Not tested.

[d] HSR*myc* was tested both as the λ clone and as a plasmid subclone.
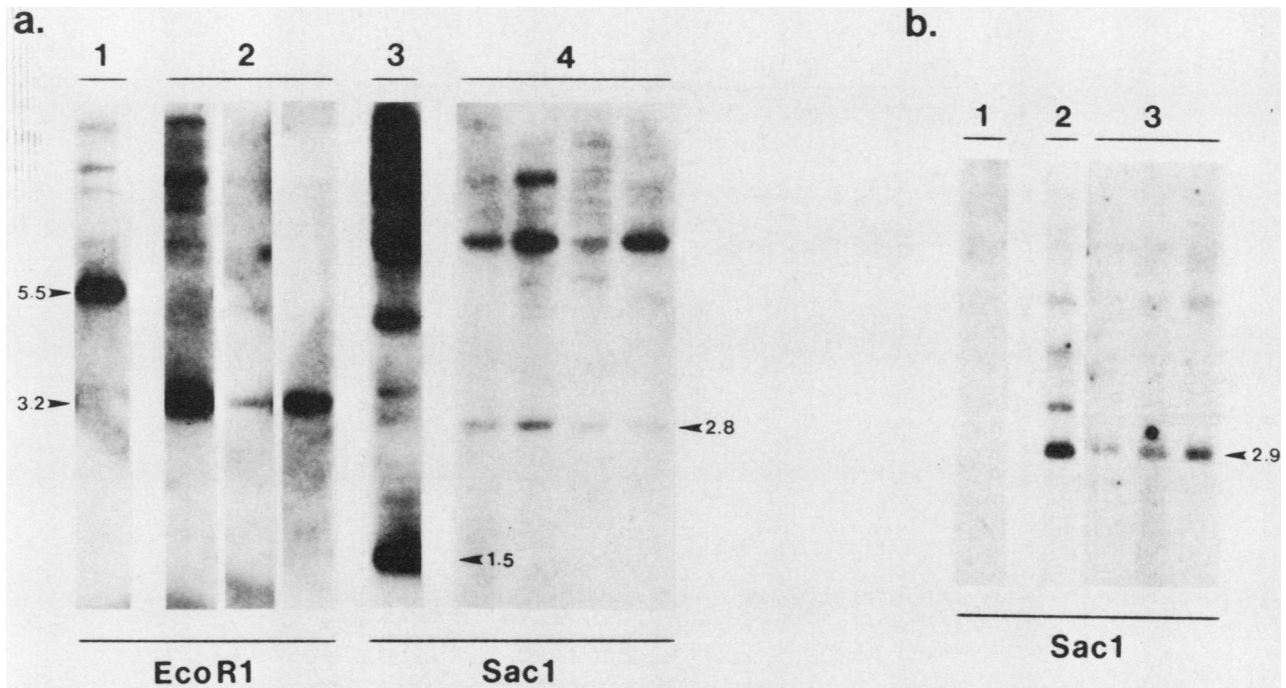
[e] DM*myc* was tested as the λ clone.

FIG. 4. Presence of *myc* alleles and EJ*ras* in REC transformed by cotransfection. DNA (10 μg) isolated from transformed REC was digested with restriction enzymes, electrophoresed in an agarose gel, transferred to nitrocellulose filters, and probed for *myc* and *ras*. (a) *myc* DNA. The following sources of DNA were used: lane 1, REC transformed with MC29 DNA (including an LTR and v-*myc*) and EJ*ras*; lanes 2, REC transformed with LL4*myc* and EJ*ras*; lane 3, REC transformed with MLV LTR-HSR*myc* and EJ*ras*; lanes 4, REC transformed with MLV LTR-DM*myc* and EJ*ras*. Lanes 1 and 2 were analyzed with ^{32}P-labeled v-*myc* probe, and lanes 3 and 4 were analyzed with human c-*myc* exon 2 probe. Each lane contained DNA from a separate colony of transformed cells. (b) *ras* DNA. The following sources of DNA were used: lane 1, normal REC; lane 2, REC transformed with LL4*myc* and EJ*ras*; lanes 3, REC transformed with MLV LTR-DM*myc* and EJ*ras*. All lanes were tested with a ^{32}P-labeled v-Ha-*ras* probe. The lengths (in kilobases) of diagnostic restriction fragments are indicated.

genes and cloned DNA of MC29 virus were also tested for their ability to cooperate with EJ*ras* in inducing focus formation in REC.

Table 1 shows the results of these assays. As a positive control, we confirmed the previous findings of Land et al. (28), who found that the v-*myc* gene in MC29 DNA, which was transcribed from the retroviral LTR, cooperated with EJ*ras* and produced transformation of REC. Only one of the c-*myc* alleles that we tested, LL4*myc*, exhibited cotransformational ability. In repeated experiments, 25 to 100 foci were obtained by using 6 μg of EJ*ras* plasmid and an equimolar amount of LL4*myc* plasmid transfected onto plates containing approximately 5 × 10⁵ to 10⁶ REC. Repeated attempts to cotransform REC with EJ*ras* and c-*myc*(ch), LL3*myc*, or LL6*myc* failed, as did attempts to cotransform REC with EJ*ras* and co-*myc*(hu), HSR*myc*, or DM*myc*. Neither LL4*myc* nor EJ*ras* alone induced foci in REC. We also used the chicken B-*lym* clone and found that neither by itself nor in combination with LL4*myc* or with EJ*ras* did it produce foci in REC (data not shown).

We characterized the REC transfected with EJ*ras* and the various c-*myc* alleles by assessing their growth characteristics and tumorigenicity. In culture, only cells transformed with LL4*myc* and EJ*ras* displayed transformed morphology and a focal pattern of growth. These cells were readily cloned and became established lines of overtly transformed cells that grew to high densities. Plates of cells transfected with other c-*myc* alleles and EJ*ras* did not appear transformed and grew as flat monolayers. Furthermore, the growth and proliferation of cells from these cultures de-

clined, and they appeared to reach a "crisis" in a manner similar to that displayed by untransfected REC.

The tumorigenicity of cells transfected with various c-*myc* alleles and EJ*ras* was assessed by subcutaneous injection of these cells into irradiated, nude mice (Table 1). When 10⁴ to 10⁶ cells grown from individual foci or confluent monolayers transfected with LL4*myc* and EJ*ras* were injected into nude mice, tumor masses consistently developed, reached diameters of 2 to 3 cm or more, and eventually caused death. However, when cells transfected with the other c-*myc* alleles and EJ*ras* were injected into these animals, no tumors resulted.

Presence and transcription of LL4*myc* and EJ*ras* in transformed REC. To demonstrate that the transformed colonies arising after transfection with LL4*myc* and EJ*ras* were indeed the recipients of these oncogenes, we analyzed the DNAs and RNAs from several such colonies for the presence (Fig. 4) and expression (Fig. 5) of the transfected genes. *Eco*RI digestion of transformed REC DNA generated a LL4*myc*-specific 3.2-kb fragment (Fig. 4a, lanes 2), which was not present in DNA from REC transformed by v-*myc* and EJ*ras* (Fig. 4a, lane 1) or normal REC (data not shown); *Sac*I digestion produced a human c-Ha-*ras*1 (EJ*ras*)-specific 2.9-kb fragment (Fig. 4b, lane 2), which was not present in the DNA of normal REC (Fig. 4b, lane 1). In cells transformed by MC29 v-*myc* and EJ*ras*, analysis of *Eco*RI-digested DNA revealed a 5.5-kb *myc*-specific band, as expected for the transfected MC29 LTR-v-*myc* gene (Fig. 4a, lane 1). The intensities of the bands detected with the *myc* probe varied considerably among the different clones of
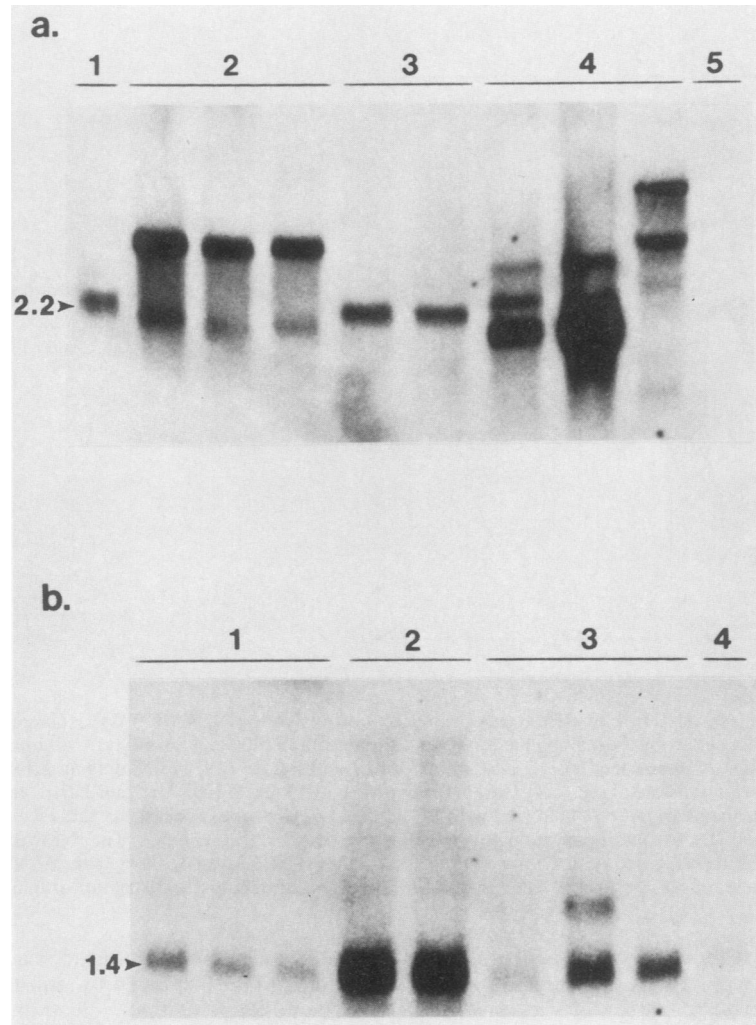
FIG. 5. Expression of *myc* alleles and EJ*ras* in REC transformed by cotransfection with these two genes. Poly(A)$^+$ RNA (5 μg) was isolated from transformed REC, electrophoresed in formaldehyde-agarose gels, transferred to nitrocellulose filters, and probed for *myc* and *ras*. Each lane contained RNA from a separate colony of transformed cells. (a) *myc* RNA. The following sources of RNA were used: lane 1, COLO320 HSR*myc* tumor cell line; lanes 2, REC transformed with MLV LTR-DM*myc* and EJ*ras*; lanes 3, REC transformed with MLV LTR-HSR*myc* and EJ*ras*; lanes 4, REC transformed with LL4*myc* and EJ*ras*; lane 5, normal REC. Lanes 1, 2, 3, and 5 were analyzed with ^{32}P-labeled human c-*myc* exon 2 probe, and lanes 4 were analyzed with ^{32}P-labeled v-*myc* probe. (b) *ras* RNA. The following sources of RNA were used: lanes 1, REC transformed with MLV LTR-DM*myc* and EJ*ras*; lanes 2, REC transformed with MLV LTR-HSR*myc* and EJ*ras*; lanes 3, REC transformed with LL4*myc* and EJ*ras*; lane 4, normal REC. The filter was annealed with ^{32}P-labeled v-Ha-*ras* probe.

EJ*ras*- and LL4*myc*-cotransformed REC, indicating the variable number of copies of LL4-*myc* DNA incorporated into different transfectants. These results also indicated that many copies of LL4*myc* were not required to cotransform REC.

Both *myc* and *ras* were expressed in the transformed cells (Fig. 5a, lanes 4 and Fig. 5b, lanes 3). The levels of expression of both oncogenes were variable among different transformed REC lines, and in the case of one line longer novel LL4*myc* transcripts were detected. In all cases, however, the level of expression of *myc* appeared to be far above the level found in normal REC (Fig. 5a, lane 5), whereas the level of expression of EJ*ras* was slightly above the level of expression of endogenous c-*ras* genes observed in normal REC (Fig. 5b, lane 4). DNA from tumors in nude mice caused by these cells was examined and showed no further amplification of either the *myc* gene or the EJ*ras* gene. An analysis of the RNA from these tumors revealed that the levels of *myc* and *ras* expression were comparable to levels in the parental cells in culture (data not shown).

Transcription of c-*myc* alleles introduced into established lines of rat cells. One possible explanation for the cotransformation activity of LL4*myc* and the inactivity of the other alleles is that only the LL4*myc* clone was efficiently expressed following transfection of REC. To compare the transcription of LL4*myc* with that of the other alleles, we introduced the various c-*myc* genes into Rat-2 cells by cotransfection with a plasmid containing the herpes simplex virus thymidine kinase gene and selection in medium containing hypoxanthine, aminopterin, and thymidine. Colonies of tk$^+$ Rat-2 cells bearing the transfected c-*myc* alleles were identified by analyzing cellular DNA. Poly(A)$^+$ RNAs from these colonies were then examined for *myc* transcripts by gel electrophoresis and the Northern blotting procedure (Fig. 6).
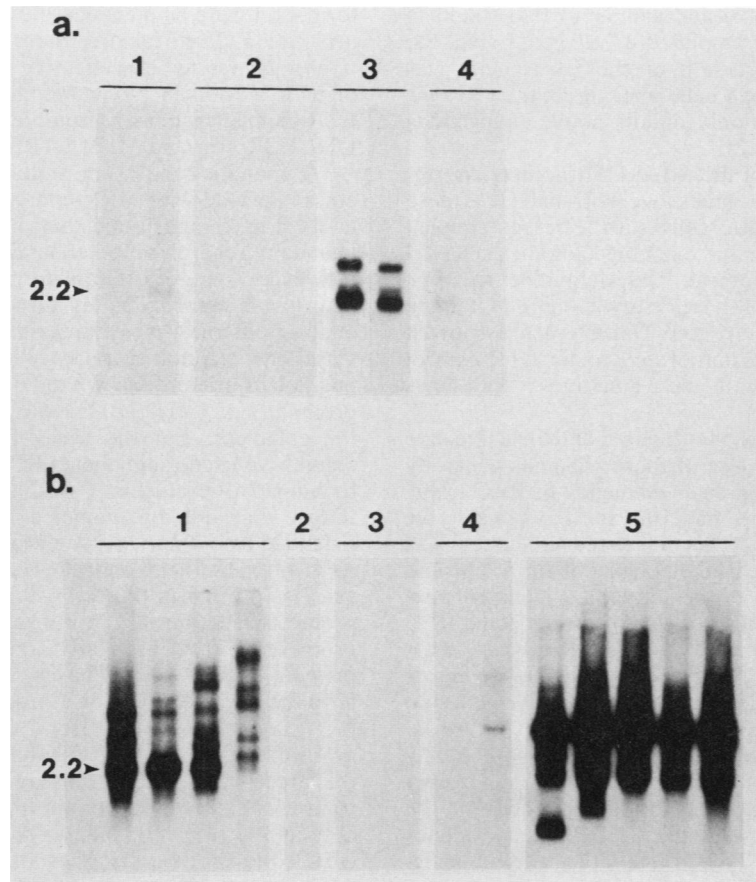
FIG. 6. Comparison of expression of different c-myc alleles and recombinant c-myc clones in Rat-2 cells. Poly(A)$^+$ RNA was prepared from Rat-2 cells cotransfected with c-myc alleles and a plasmid containing the viral thymidine kinase gene, electrophoresed in formaldehyde-agarose gels, transferred to nitrocellulose filters, and probed for myc expression. Each lane contained 5 µg of RNA from a separate tk$^+$ colony shown to contain multiple copies of cotransfected c-myc DNA. (a) RNAs from Rat-2 cells cotransfected with avian myc alleles. Lanes 1, c-myc(ch); lanes 2, LL3myc; lanes 3, LL4myc; lanes 4, LL6myc. Filters were probed with ^{32}P-labeled v-myc probe. (b) RNAs from Rat-2 cells cotransfected with human myc alleles and recombinant c-myc clones. Lanes 1, MLV LTR-HSRmyc; lane 2, HSRmyc; lanes 3, c-myc(hu); lanes 4, DMmyc (λ clone); lanes 5, MLV LTR-DMmyc. Filters were annealed with ^{32}P-labeled human c-myc exon 2 probe.

We found that LL4myc was transcribed in Rat-2 cells (Fig. 6a, lanes 3) to a much greater extent than the other, cotransformationally inactive chicken (Fig. 6a, lanes 1, 2, and 4) and human (Fig. 6b, lanes 2, 3, and 4) c-myc alleles and that the levels of LL4myc RNA in Rat-2 cells were similar to those in cotransformed REC. Efficient expression of LL4myc was not due to a higher number of copies of this allele in Rat-2 cells, since genomic blots of DNAs from Rat-2 cells containing the various chicken and human c-myc genes revealed comparable numbers of copies of the different transfected alleles (data not shown). Instead, the abundance of LL4myc RNA was most likely due to the favorable position and orientation of the ALV LTR within LL4myc.

Increasing COLO320 myc transcription by the Mo-MLV LTR. The results described above suggested that the level of transcription of c-myc alleles may be the important determinant of REC cotransforming activity. Certainly, the mere presence of a truncated c-myc gene (as in LL3myc and DMmyc) or of a c-myc gene with an altered coding sequence (as in LL3myc) is insufficient for cotransformation of REC with EJras.

If the level of myc transcription is an important determinant of REC cotransforming activity, then increasing the expression of a cotransformationally inactive c-myc allele might confer such activity. Therefore, we placed both of the COLO320 c-myc genes, which had no cotransforming activity, under the control of Mo-MLV transcriptional enhancer-promoter elements (Fig. 2). MLV LTR-HSRmyc contained only a portion of the LTR (part of U3 and R), whereas MLV LTR-DMmyc contained the entire LTR plus approximately 1 kb of gag. Both plasmids contained the LTR enhancer and promoter, whereas only the second contained a splice donor site. Both of these recombinant genes cooperated with EJras and cotransformed REC (Table 1). The efficiencies of MLV LTR-HSRmyc and MLV LTR-DMmyc in transforming REC with EJras were not significantly different from each other, but were consistently greater than that of LL4myc. Cells cloned from these foci grew as transformed lines and were tumorigenic in nude mice, even when as few as 10^2 cells were injected. An analysis of the DNAs obtained from the transformed REC cells demonstrated the presence of both the recombinant myc (Fig. 4a, lanes 3 and 4) and EJras genes (Fig. 4b, lanes 3; data not shown for cells transformed by MLV LTR-HSRmyc and EJras). An analysis of the RNA showed that the recombinant COLO320 myc genes were efficiently expressed at a level far above that found in normal

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REC (Fig. 5a, lanes 2 and 3) and similar to that found in COLO320 cells with highly amplified c-*myc* genes (Fig. 5a, lane 1). The level of expression of the EJ*ras* gene was variable in the cotransformed cells and, in contrast to *myc* expression, may have been only slightly above endogenous levels (Fig. 5b, lanes 1 and 2).

To determine the effect of the MLV LTR upon transcription of the COLO320 *myc* genes, we cotransfected these recombinant genes into Rat-2 cells with a herpes simplex virus thymidine kinase gene and examined colonies selected with hypoxanthine, aminopterin, and thymidine for *myc* expression. A comparison of these cells with cells containing the native COLO320 HSR*myc* and DM*myc* genes showed markedly augmented expression of *myc* under LTR control (Fig. 6b, compare lanes 1 with lane 2 and lanes 5 with lanes 4).

We made vectors containing both EJ*ras* and an active *myc* gene on the same plasmid. Use of these plasmids generally resulted in two- to fivefold greater efficiency of REC transformation compared with use of EJ*ras* and the same active *myc* on separate plasmids. This increased efficiency was presumably due to the increased efficiency of single plasmid transfections or to the LTR of the active *myc* allele enhancing expression of EJ*ras* or both. We also cotransfected REC with chicken B-*lym* plus MLV LTR-HSR*myc* or MLV LTR-DM*myc* and did not obtain foci. In addition, vectors containing both the B-*lym* gene and the active *myc* genes on the same plasmid failed to produce foci in REC.

Substitution of normal chicken and human c-*myc* coding sequences for the tumor *myc* sequence did not affect cotransforming activity. The nucleotide sequences of the coding regions of LL4*myc* and the COLO320 *myc* alleles have not been determined. To be certain that altered nucleotide sequences in these alleles are not important for the REC cotransforming activity of these genes under retroviral LTR control, we substituted the coding portions of the normal chicken and human *myc* genes for the equivalent domains of LL4*myc* and MLV LTR-HSR*myc* (Fig. 2). These constructions [LL4 LTR-c-*myc*(ch) and MLV LTR-c-*myc*(hu)] were efficiently transcribed when they were introduced into Rat-2 cells (data not shown). When used to cotransform REC, both were as efficient in cooperating with EJ*ras* to produce foci as the equivalent tumor-derived *myc* alleles (Table 1). Cells from these foci were indistinguishable from cells transformed with the tumor-derived *myc* alleles in terms of morphology, growth, and, in the case of REC cotransformed with MLV LTR-c-*myc*(hu), tumorigenicity.

Activation of normal human c-*myc* gene by the MLV LTR enhancer element. In all of the cotransformationally active c-*myc* clones described above, the retroviral LTRs were positioned so that c-*myc* transcription could be initiated within the LTRs, which thus acted as promoters and produced structurally abnormal c-*myc* mRNAs. We wanted to determine whether LTRs could also activate c-*myc* genes by virtue of their enhancer function alone, thereby producing normal c-*myc* transcripts at elevated levels. Therefore, we used a portion of the Mo-MLV U3 region extending from a *Hin*dIII site in the 5' flanking region to the *Sac*I site within U3 (24) to enhance the expression of the normal human c-*myc* gene. This fragment was cloned in both orientations with respect to the gene into the *Hin*dIII site approximately 2.5 kb 5' to the first c-*myc* exon, into the *Pvu*II site about 0.4 kb 5' to the first c-*myc* exon, or into the *Eco*RI site about 0.6 kb 3' to the third c-*myc* exon (Fig. 3). When these recombinants were cotransfected into REC with EJ*ras*, they all yielded foci of transformed cells. The efficiencies of focus

formation were similar regardless of enhancer placement or orientation. Furthermore, these enhanced c-*myc* constructions appeared to be as effective in promoting transformation of REC as human c-*myc* recombinants in which the MLV LTR element acts as a promoter [MLV LTR-DM*myc*, ML LTR-HSR*myc*, and MLV LTR-c-*myc*(hu)].

We examined the expression of human c-*myc* in the REC cotransformed with MLV-enhanced human c-*myc* recombinants (Fig. 7) and found that *myc* transcripts were usually abundant regardless of enhancer position or orientation. However, some of the transformed REC did not have very high levels (approximately three to five times the level of endogenous rat c-*myc* transcripts [data not shown]). These variations did not correlate with any particular enhancer position or orientation. We did observe that usage of the two promoters of c-*myc*(hu) (5) was affected by the position of the enhancer. For the assay for c-*myc* mRNA in these studies we used radiolabeled RNA that was complementary to human c-*myc* exon 1 mRNA and was protected from RNase digestion by human c-*myc* transcripts. The probe extended from the *Pvu*II site near the 3' end of exon 1 to the *Xho*I site near the 5' end; the region encompassing the *Xho*I site was present in transcripts originating from the c-*myc* 5' promoter (P1), but not in transcripts originating from the 3' promoter (P2). Thus, c-*myc* transcripts initiated at P2 protected a fragment of 349 bases, whereas transcripts initiated 5' to the *Xho*I site of exon 1 protect a fragment of 440 bases (Fig. 7A). Transcripts from REC cotransformed by c-*myc*(hu) with a 5' MLV enhancer produced predominantly the larger fragment in our assay, indicating that most of the transcripts initiated upstream from the *Xho*I site (i.e., probably at P1) (Fig. 7B, lanes a through d). Transcripts from REC cotransformed by c-*myc*(hu) with a 3' MLV enhancer tended to produce more of the shorter fragment in our assay, indicating more frequent use of P2 (Fig. 7B, lanes e).

DISCUSSION

Assays for c-*myc* alleles. We began the studies described above in order to determine functionally important features of abnormal c-*myc* genes cloned from bursal lymphomas and human tumor lines. Although c-*myc* is among the most widely studied of the protooncogenes, largely because it has been implicated as a contributor to neoplasia in so many contexts (see Introduction), there are as yet few methods for systematic analysis of the oncogenic potential of activated *myc* alleles. None of the previously described biochemical features of c-*myc* protein—localization in the nucleus (2, 21, 22, 39), copurification with components of the nuclear matrix (13), binding to total cellular DNA (37), or activation of heterologous promoters in *trans* (26)—has yet been rigorously associated with the normal or neoplastic functions of various *myc* genes. Furthermore, among the available *myc* alleles, only v-*myc* genes in retroviral genomes manifest a readily observed, single-step oncogenic effect in cell culture. Biological assays for c-*myc* oncogenes have required conditions in which a second oncogene is present (28), only a subtle component of the transformed phenotype is measured (e.g., immortalization or growth in growth factor-deficient medium [4, 25]), or the endpoint is tumorigenesis in whole animals (25).

LL4*myc* is a cotransformationally active, natural allele. Among the available assays for c-*myc*, REC cotransformation with a mutant c-Ha-*ras* gene (EJ*ras*) is simple, rapid, and semiquantitative. However, previous positive results with this assay have been obtained with a translocated c-*myc* gene cloned from a mouse plasmacytoma which had been
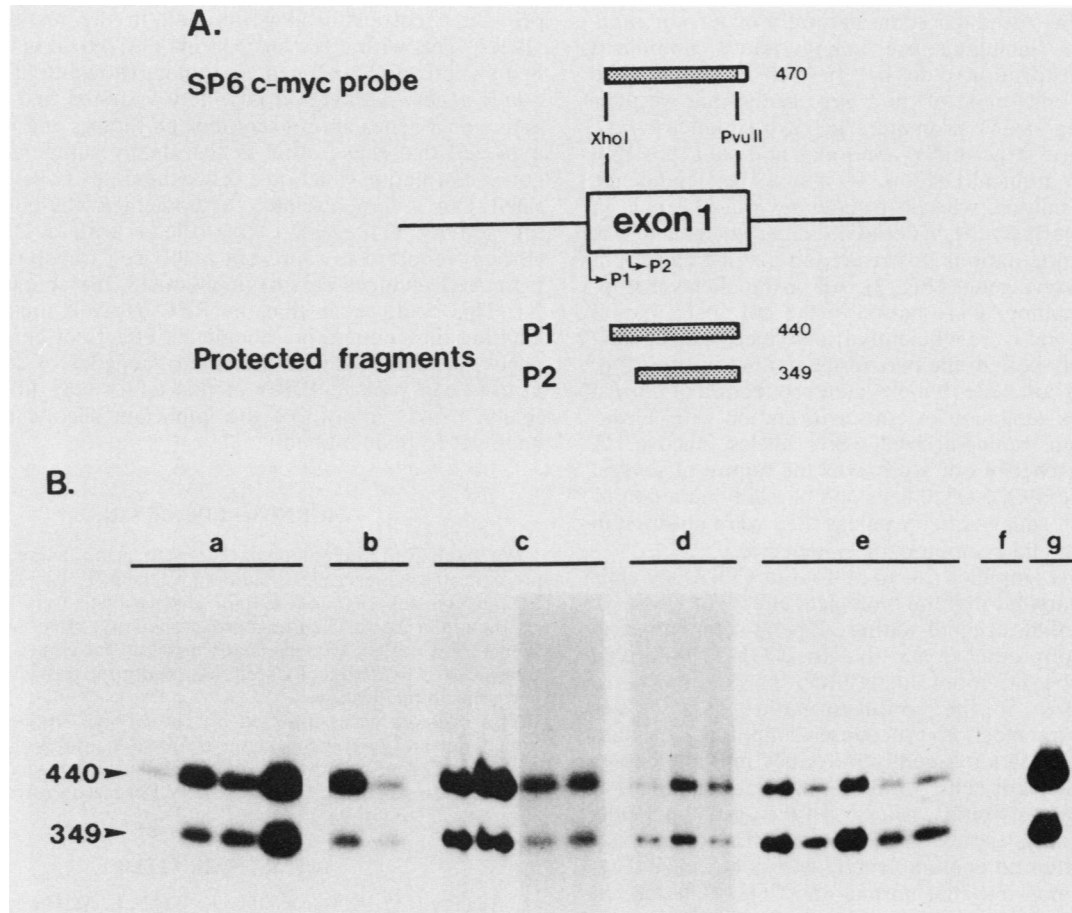
FIG. 7. Analysis of *myc* transcripts from REC transformed with Mo-MuLV-enhanced human c-*myc* recombinants and EJ*ras*. (A) Diagram of human c-*myc* exon 1 with its dual promoters (P1 and P2). The SP6 transcript complementary to c-*myc* exon 1 is shown, along with the fragments protected from RNase digestion by hybridization to c-*myc* transcripts originating at P1 and P2. Note that protection of a fragment of 440 bases indicates the presence of a transcript initiated at or 5′ to the *XhoI* site and not necessarily at P1. (B) Fragments of the SP6 c-*myc* RNA probe protected from RNase digestion by 10 μg of total cellular RNA isolated from REC transformed with EJ*ras* and different c-*myc* constructions shown in Fig. 3. The following sources of RNA were used: lanes a, REC transformed by Enh-c-*myc*(hu) 1a; lanes b, REC transformed by Enh-c-*myc*(hu) 1b; lanes c, REC transformed by Enh-c-*myc*(hu) 2a; lanes d, REC transformed by Enh-c-*myc*(hu) 3a; lanes e, REC transformed by Enh-c-*myc*(hu) 3b; lane f, normal REC; lane g, Rat-1 cells transfected with MLV LTR-c-*myc*(hu).

manipulated in vitro and placed under the control of a heterologous promoter (28); moreover, it was not known whether the gene encodes an entirely normal c-*myc* protein, and transformed cells were not assayed for persistence and expression of the c-*myc* oncogene. Most of the tumor-derived alleles which we examined also failed to display cotransformation activity in the REC assay unless they were further manipulated. But LL4*myc*, a bursal lymphoma allele with a solitary ALV LTR in the first c-*myc* intron in the same transcriptional orientation as c-*myc*, was active without further alterations of the molecular clone. Although the coding region for c-*myc* protein in LL4*myc* has not been sequenced, we were able to show that the activity of this gene was independent of any amino acid changes. We exchanged the coding exons of normal chicken c-*myc* for those of LL4*myc* without loss of co-transformation efficiency (Fig. 2 and Table 1). Thus, quantitative changes in the expression of chicken c-*myc* appeared to be sufficient for the cotransforming property of LL4*myc*.

Augmented expression of c-*myc* is sufficient and probably necessary for cotransformation activity. We extended the conclusions drawn from studies with LL4*myc* in several

ways. (i) Introduction of biologically active or inactive c-*myc* alleles into an established line of rat fibroblasts revealed that efficient production of c-*myc* RNA correlated directly with cotransforming activity in embryo cells (Fig. 6 and Table 1). (ii) Placement of functionally competent retroviral enhancers or promoters within or adjacent to c-*myc* genes, both normal and tumor derived, rendered the alleles competent in the cotransformation assay (Table 1). (iii) All cotransformed REC lines contained abundant amounts of *myc* RNA and variable amounts of *myc* DNA, in addition to EJ*ras* DNA and RNA (Fig. 4 and 5). In addition, several of these lines have been shown to produce high levels of c-*myc* protein (39). In sum, our findings provide biological and biochemical support for the notion that all c-*myc* genes examined to date require enhanced levels of expression to contribute to the cotransformed phenotype; moreover, no changes in the coding exons are necessary, implying that the alterations in expression are sufficient. However, we cannot exclude the possibility that certain qualitative changes in c-*myc* protein would suffice to cotransform cells in the absence of quantitative effects.

Changes in the structure of c-*myc* RNA are not required for

cotransformation. Although some naturally occurring alterations of c-myc (including insertion mutations, amplifications, and translocations) do not perturb the immediate transcriptional unit, most of the c-myc genes that we activated by adding strong promoters in vitro produce c-myc mRNAs that are structurally abnormal and thus possibly translated in an atypical fashion. To assess the significance of such abnormalities, we constructed several plasmids in which a promoterless, MLV-derived enhancer element was placed in both orientations upstream and downstream from intact human c-myc genes (Fig. 3). All of the alleles manipulated in this manner were active in the cotransformation assay (Table 1) and were efficiently transcribed from at least one and probably both of the two normal initiation sites (Fig. 7) (5). Thus, we conclude that elevated production of normal c-myc mRNA is sufficient for cotransformation with EJras.

Why are some tumor-derived c-myc alleles inactive? A disappointing aspect of our study was the failure of several c-myc clones from COLO320 cells and bursal lymphomas to display cotransforming activity unless they were outfitted in vitro with additional promoters or enhancers.

Since c-myc is amplified 20- to 50-fold in COLO320 cells (2), it might be argued that the biological effects of HSRmyc and DMmyc in their original settings depend solely upon an expansion in copy number and that the COLO320-derived alleles would be no more likely than normal c-myc to supplement EJras in the cotransformation assay. Conversely, since transfection procedures employing calcium phosphate precipitates frequently introduce multiple copies of DNA into recipient cells, we might have anticipated that normal c-myc genes would cotransform the occasional cells that acquire many copies. REC lines cotransformed by active alleles often do contain several copies of c-myc (Fig. 1), so it seems possible that normal and COLO320-derived alleles are transcribed at less than the expected efficiency in REC after transfection.

The failure of clones LL6myc and LL3myc to cotransform REC is presumably due either to the absence of a critical component of the original locus in the molecular clones or to relatively poor enhancer function of the ALV LTR in rat cells. Clone LL3myc contains only the 5' portion of the ALV provirus; thus, although it harbors an intact LTR, it lacks a region adjacent to the 3' LTR to which some enhancer activity has been assigned (27, 30). Clone LL6myc includes all of the proviral DNA in the original locus, but it is possible that some sequences flanking the cloned domain in the chicken chromosome are required for the augmented expression previously documented with tumor RNA (36).

Are structural lesions in c-myc significant? Several of the tumor-derived c-myc alleles bear structural alterations, including rearrangements that displace the first exon (DMmyc and LL3myc) or base substitutions that alter the c-myc coding sequence (LL3myc). Our finding that cotransformation of REC requires only quantitative changes does not refute the possibility that these additional mutations are relevant to tumorigenesis. Development of a tumor in an intact animal is a more complex process than transformation of a cultured cell, and it is likely that diverse alterations of c-myc can confer growth advantage to cells in vivo; this seems the best way at present to account for the clonal dominance of tumor cells bearing several changes in c-myc. The absence of suitable assays limits our appreciation of the biological effects of such changes.

Can oncogenes other than activated rac genes cooperate with myc in cotransformation assays? In the experiments described above, we depended exclusively upon EJras to provide a cotransforming function in the testing of myc alleles. Yet, with a few exceptions (33, 49), it is unusual to find an activated c-ras gene in tumors shown to have altered c-myc genes. Although it is widely assumed that additional active oncogenes are present in such tumors and it might be expected that these other genes ideally supplement c-myc cotransformation functions, few candidates have been identified. Our several attempts to supplement the cotransforming activity of certain c-myc alleles with a DNA clone (B-lym) reported to represent a 3T3 cell transforming gene from ALV-induced bursal lymphoma (8, 16) were unsuccessful. This could mean that the REC assay is unsuitable for eliciting the cooperative noeplastic effects of certain oncogenes, despite evidence that the oncogenes of some DNA viruses can replace EJras in this assay (28, 40). Alternatively, it may imply that the important second oncogenes have yet to be identified.

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