# Isolation and Characterization of c-myc, a Cellular Homolog of the Oncogene (v-myc) of Avian Myelocytomatosis Virus Strain 29

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The chicken genome contains nucleotide sequences homologous to the transforming genes (oncogenes) of a number of avian retroviruses. We have isolated chicken DNA (c-myc) that is homologous to the oncogene (v-myc) of the avian myelocytomatosis virus MC29 and have compared the structures of the cellular and viral genes. Results from restriction endonuclease mapping of c-myc and from analysis of heteroduplexes between the DNAs of the cellular and viral genes show that c-myc is homologous to 1,500 nucleotides in v-myc DNA. This homologous region is interrupted in c-myc by an intron-like sequence of 1,100 nucleotides which is absent from v-myc. Nuclear RNA from normal chicken cells contains at least five species of transcripts from c-myc ranging from 2.5 to 6.5 kilobases in length. By contrast, cytoplasm contains only the 2.5-kilobase c-myc RNA. These features of the c-myc gene and its nuclear transcripts are characteristic of normal cellular genes and suggest that the myc gene is of cellular rather than viral origin. The exons in c-myc may define two functional domains in the gene and may therefore facilitate the dissection of the different oncogenic potentials of the MC29 virus.

The avian retrovirus MC29 induces a wide spectrum of neoplastic diseases in chickens, including renal and hepatic carcinomas, sarcomas, and leukemias; in vitro the virus transforms macrophages, fibroblasts, and epithelial cells (9). The oncogenic potentials of the virus are thought to be encoded in a single gene, located approximately in the middle of the 5.5kilobase (kb) viral genome. The virus probably contains no other functional genes; only portions of the gag and env genes are present, and pol is deleted entirely (7, 12, 23). Consequently, the virus is defective in replication and requires a helper virus to propagate. The oncogene of MC29 is homologous to the oncogenes of three other related but distinct retroviruses (MH2, CMII, OK10) that have oncogenic properties similar to those of MC29 (9). This common oncogene has been denoted viral myc (v-myc).

The v-myc genes have been estimated to consist of 1,500 to 2,000 nucleotides (7, 12, 23), but they are fused to the defective gag gene in MC29, MH2, and CMII, giving rise to hybrid proteins with gag and myc determinants. By contrast, v-myc in OK10 may not be fused to gag and is instead expressed by a subgenomic mRNA (2). The oncogene v-myc is homologous to a cellular gene (c-myc) that is present in several avian and mammalian species (20, 22) and has apparently been conserved during the course of vertebrate evolution. This cellular gene is transcribed in all vertebrates that have been tested. The final product of transcription is a polyadenylated RNA 2.5 kb in length (24).

Chickens infected with avian leukosis virus develop bursal tumors after long latency periods. It was shown recently that the avian leukosis virus DNA in most of these tumors is integrated in the vicinity of c-myc, and as a possible consequence of the integration, the expression of c-myc is greatly enhanced (11, 15, 18). It therefore appears likely that the myc gene can exert an oncogenic effect as either a viral or cellular gene, but the function of the protein encoded by myc has not been determined, and the role (if any) of c-myc in the economy of normal cells is not known.

In this communication we describe the isolation and characterization of DNA encompassing most, if not all, of the cellular *myc* gene. The results obtained by restriction enzyme mapping and by electron microscopy of heteroduplexes between viral and cellular *myc* suggest that *cmyc* may contain an intron in the middle of the gene; by contrast, there is no intron in the virusencoded homolog. Furthermore, we demonstrate that *c*-*myc* is transcribed in the nucleus into large RNAs, which appear to be processed to one cytoplasmic mRNA. These findings imply that c-myc contains further introns outside of the region of homology with v-myc.

### MATERIALS AND METHODS

Isolation of DNA. Chicken DNA was extracted from 10-day-old embryos obtained from H & N Farms, Redmonton, Wash. Embryos were homogenized in 0.1 M NaCl-20 mM Tris-hydrochloride (pH 7.4)-10 mM EDTA. Pronase was added to 500  $\mu$ g/ml, sodium dodecyl sulfate was added to 0.5%, and hydrolysis was carried out for 2 h at 37°C. DNA was isolated by repeated extraction with phenol-chloroform (50:50) and precipitated with ethanol.

MC29 DNA was obtained from the chimeric lambda phage  $\lambda$ MC38 previously described (28). The phage was grown in liquid cultures, and phage DNA was purified as previously described (1). The MC29 DNA was excised from the chimeric phage DNA with the restriction endonuclease *Eco*RI and was isolated by sucrose gradient centrifugation.

Lambda phages carrying the cellular myc sequence were isolated from the chicken DNA library described in reference 6. This library was constructed by partially digesting chicken DNA with *HaeIII* and *AluI*, adding *Eco*RI linkers to the blunt-ended DNA, and cloning the resulting fragments into the *Eco*RI site of lambda phage Charon 4A. A 9.4-kilobase-pair (kbp) *Bam*HI fragment from one recombinant phage,  $\lambda c$ *myc2*, was subcloned into the *Bam*HI site of the plasmid pBR322 and was designated pC-*myc*. The plasmid was propagated in *Escherichia coli* strain HB101 and was amplified by using chloramphenicol as an inhibitor of protein synthesis. Plasmid DNA was isolated as previously described (25).

Nucleic acid hybridizations. A 1.5-kbp PstI fragment of the cloned MC29 DNA was used as a viral myc probe. The fragment, which was excised from a subclone in the PstI site of pBR322 before use, encodes most of the viral myc gene; only a small portion of the 5'-proximal sequences of v-myc are missing from the fragment (28). In experiments designed to determine the orientation of the cellular myc gene in chromosomal DNA, the viral PstI fragment was cleaved with SalI to yield two fragments, 0.6 and 0.9 kbp in length, which originate from the 5'- and 3'-terminal halves of v-myc, respectively (28). Fragments were purified by sucrose gradient centrifugation or agarose gel electro-phoresis and were labeled in vitro with  $\alpha$ -<sup>32</sup>P-nucleoside triphosphates by using avian myeloblastosis virus reverse transcriptase and oligomers of calf thymus DNA as primers (13). Chicken chromosomal DNA or recombinant DNAs to be hybridized with labeled probes were cleaved with endonucleases; the resulting fragments were separated by electrophoresis in agarose gels and transferred to sheets of nitrocellulose. The gels were calibrated as previously described (26). Filter hybridizations were carried out at 41°C in a buffer containing 50% formamide as previously described (21).

**Electron microscopy.** Purified MC29 and  $\lambda c$ -myc2 DNAs were mixed, denatured with alkali, neutralized, and annealed at 25°C in 50% formamide; heteroduplexes were mounted for electron microscopy as described in reference 5.

Analysis of RNA. Nuclear and cytoplasmic polyad-

enylated RNA was purified from 10- to 13-day-old White Leghorn chicken embryos (Kragga, Sweden) as described previously (28). RNA was analyzed by electrophoresis in denaturing agarose gels containing 2.2 M formaldehyde and was transferred to nitrocellulose sheets (27) for hybridization with radiolabeled probes.

## RESULTS

Molecular cloning of the cellular myc gene. An analysis of the structure and topography of cmyc necessitated the molecular cloning of the locus. A chicken genomic DNA library amplified in the lambda vector Charon 4A (6) was screened with v-myc probe. Four phages with identical inserts were isolated: each contained two EcoRI fragments of 10 and 5 kbp in size, of which only the larger fragment carried myc sequences (data not shown).

One clone,  $\lambda c$ -myc2, was digested with BamHI, and a 9.4-kbp fragment was subse-



FIG. 1. Comparison of restriction fragments from chromosomal and cloned DNAs carrying c-myc. Chicken chromosomal and plasmid pC-myc DNAs were cleaved with three different restriction endonucleases. The resulting fragments were separated in an agarose gel, transferred to a nitrocellulose filter, and hybridized with v-myc probe. Lanes: 1 and 2, PvuIIcleaved chromosomal and plasmid DNA, respectively; 3 and 4, BamHI-cleaved chromosomal and plasmid DNA, respectively; 5 and 6, SacI-cleaved chromosomal and plasmid DNA, respectively. Vol. 42, 1982

quently subcloned in pBR322; the chimeric plasmid was denoted pC-myc. To demonstrate that no major rearrangements occurred in c-myc during molecular cloning, chicken chromosomal and pC-myc DNAs were cleaved with restriction enzymes, and the sizes of the resulting fragments were compared by separation in agarose gels followed by transfer to nitrocellulose sheets and hybridization with v-myc probe. Figure 1 shows that the PvuII (lanes 1 and 2), BamHI (lanes 3 and 4), and SacI (lanes 5 and 6) fragments produced from the cloned DNA were identical in length to those from chicken chromosomal DNA. We therefore conclude that the c-myc locus has not been perturbed by major changes during the cloning and amplification procedures.

Heteroduplex analysis of c-myc. An analysis of heteroduplex molecules formed between the  $\lambda c$ -myc2 and the cloned EcoRI-cleaved MC29 DNA was carried out. The electron micrograph shown in Fig. 2 demonstrates that the  $\lambda c$ -myc DNA forms two duplex regions with the cloned MC29 DNA (0.70 and 0.84 kbp in length) and that these domains are interrupted by a 1.1-kbp segment of DNA which is unrelated to the MC29 DNA. In the MC29 DNA the duplex region is a contiguous 1.5-kbp segment of DNA that maps



FIG. 2. Electron microscopic analysis of heteroduplexes formed between  $\lambda c$ -myc2 and cloned MC29 DNA. The DNAs encode the myc sequences in the chicken and in the viral genomes. The MC29 DNA was derived from circular unintegrated MC29 DNA that was cloned in a bacterial vector by using a single EcoRI site located in the env region of MC29. The MC29 DNA used here has been excised from its bacterial vector and is therefore permuted with regard to the EcoRI site. The molecular orientation of the permuted MC29 DNA is as follows: 5' end, env (rightward part), large terminal redundancy, gag, myc, env (leftward part), 3' end.  $\Delta$  denotes deleted genes (see reference 28 for details). A, Electron micrograph; B, schematic drawing of A; C, summary of the electron microscopy data. Nine molecules were measured. a, MC29 DNA; b,  $\lambda c$ -myc2 DNA.

3.1 and 0.6 kbp from the ends of the cloned MC29 DNA. These are also the coordinates for the v-myc gene in cloned EcoRI-cleaved MC29 DNA (28); consequently, the duplex DNA formed between the two DNAs must be composed of complementary viral and cellular myc sequences.

Mapping c-myc with restriction endonucleases. A restriction map of c-myc was established by cleaving  $\lambda c$ -myc2 and pC-myc DNAs with several restriction enzymes followed by agarose gel electrophoresis, immobilization on nitrocellulose filters, and hybridization with v-mvc probe. The map illustrated in Fig. 3 shows that the myc sequences are confined to two separate regions of DNA: the location of the first domain is defined by a 0.6-kbp SacI-SalI fragment; the second domain is located in a 0.9-kbp ClaI-EcoRI fragment. The two domains are separated by a 1.1-kbp SalI-ClaI fragment that does not hybridize with the viral myc probe. This is further illustrated in Fig. 4, which shows that of the four fragments produced by cleaving pC-myc with SalI and ClaI, only the 4.8- and 3.7-kbp fragments (each encoding one of the two domains) hybridize with the v-myc probe. The 1.1kbp fragment, which maps between the two domains, does not react with the probe. (The 3.4-kbp band does not anneal with the probe because it represents a plasmid vector fragment.)

The order of the two domains with respect to the direction of v-myc gene transcription was determined by using v-myc probes that represent 5'- or 3'-terminal parts of the v-myc gene. Fragments of chicken chromosomal DNA generated with restriction endonuclease *Hin*dIII were separated in agarose gels, transferred to nitrocellulose filters, and hybridized with the two probes. The probe representing the 5'-terminal part of v-myc annealed to a 10-kbp fragment encoding the 0.6-kbp domain described above, and the 3'-terminal probe annealed to a 1.5-kbp fragment harboring the 0.9-kbp domain (data not J. VIROL.

The restriction maps are in good agreement with the results of heteroduplex analysis. It appears that c-myc contains two domains homologous to v-myc, the smaller domain located to the 5' side of the larger and the two being separated by a 1.1-kbp domain that contains no detectable homology with v-myc. The structure of the cellular myc sequences can be interpreted as if the gene contained at least two exons, 0.7 and 0.84 kbp in length, separated by a 1.1-kbp intron; the topography of c-myc is thus akin to most normal cellular genes. This interpretation is sustained by our unpublished finding that cDNA prepared with the SalI-ClaI restriction fragment that encompasses the putative intron reacts strongly with several of the precursor RNAs illustrated in Fig. 5, but very weakly with the 2.5-kb mature cytoplasmic RNA. Thus, most of the sequences represented by the SalI-ClaI restriction fragment are apparently spliced out of the RNA during its maturation. The weak reaction of the cDNA with the 2.5-kb RNA indicates that one or both of the two restriction sites (SalI and ClaI) may be located within exons rather than exactly at the junctions between the exons and the intron (Fig. 3). The matter can best be resolved by nucleotide sequencing.

The measurements of the electron micrographs suggest that c-myc occupies at least 2.6 kbp of DNA when both exons and the intron are included in the calculation, whereas restriction mapping data suggested a total of only 2.4 kbp. None of the measurements in question offer sufficient precision to make this apparent difference of any consequence.

**Transcription from c-myc.** An analysis of nuclear and cytoplasmic RNA was carried out to characterize transcription from c-myc. Polyad-enylated RNA was extracted from fractionated nuclei and cytoplasm and analyzed for c-myc



FIG. 3. Restriction map of the c-myc locus and flanking regions. Cloned c-myc DNA in  $\lambda$ c-myc2 or pC-myc was subjected to restriction enzyme analysis and hybridization with v-myc probe by the procedures of Southern (26). Mapping has not been exhaustive between the outside BamHI sites and the EcoRI sites at the ends of the clone; the EcoRI sites are shown merely to define the boundaries of the c-myc insert. The solid line represents DNA unreactive with the probe; the stippled areas denote myc-specific DNA. The letters indicate the following individual restriction enzyme cleavage sites: B, BamHI, Bg, BgII; C, ClaI; E, EcoRI; H, HindIII; K, KpnI; S, SacI; Sal, SalI.



FIG. 4. The 1.1-kbp Sall-ClaI fragment that separates the two c-myc domains does not anneal with vmyc probe. Plasmid pC-myc DNA was cleaved with BamHI, Sall, and ClaI; the resulting fragments were electrophoresed in an agarose gel immobilized on a filter and annealed with v-myc probe. Lanes: 1, stained gel; 2, autoradiogram.

RNA by electrophoresis in agarose gels followed by transfer to a nitrocellulose filter and hybridization with v-myc probe. Six RNA species (6.5, 5.0, 4.4, 3.5, 3.3, and 2.5 kb in size) were found in the nuclear fraction (Fig. 5, lane A), whereas in the cytoplasm only the 2.5-kb mRNA was detectable (Fig. 5, lane B). The data suggest that c-myc is transcribed into a nuclear precursor RNA of at least 6.5 kb in size, which is processed through intermediates into the mature 2.5kb cytoplasmic mRNA. These findings suggest that c-myc may contain introns outside of the region defined by homology with v-myc, i.e., in addition to the potential 1.1-kbp intron described above.

## DISCUSSION

Does  $\lambda c$ -myc2 contain all of the c-myc gene? The complexity of the viral myc gene in MC29 has previously been estimated to be 1,500 to 2,000 nucleotides by several experimental approaches. Our estimates of the virus-encoded gene, based here on the analysis of heteroduplexes between the viral and cellular myc genes, are in full agreement with previously published data. It remains possible, however, that the protein-coding portion of c-myc contains sequences that are not present in some of the virus-encoded genes: v-myc in MC29, CMII, and MH2 is fused to the viral gag, giving rise to hybrid proteins; some coding sequences in the 5' end (or even the 3' end) of c-myc may thus not



FIG. 5. Analysis of nuclear and cytoplasmic c-myc RNA. Polyadenylated RNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters. Hybrdization was carried out with v-myc probe. Lanes: A, nuclear RNA; B, cytoplasmic RNA.

have been acquired by the viruses. Moreover, the nucleotide complexity of c-myc has not yet been estimated by independent means, and the cell-encoded protein has not been characterized. A 2.5-kb mRNA appears to be produced from cmyc (24), but this does not establish the coding potential of the gene: large parts of the RNA may harbor sequences not utilized in translation (16). Other cellular genes homologous to retrovirus oncogenes also give rise to mRNAs which have complexities severalfold larger than those of the viral oncogene mRNAs, as exemplified by the viral and cellular src and erb genes (17a, 27a). It is possible, however, that we have isolated DNA sequences that encompass the whole c-myc transcriptional unit: the largest detectable nuclear transcript from c-myc is 6.5 kb in length, and the myc sequences in  $\lambda c$ -myc2 are flanked by enough DNA to accomodate a precursor RNA of this size. The full extent of cmyc will only be known, however, when the sites at which transcription is initiated and terminated are identified.

Nucleotide sequence of c-myc is closely related to that of v-myc. Previously published data suggested that few differences exist between the nucleotide sequences shared by v-myc and cmyc (22, 24). A comparison of the restriction endonuclease cleavage sites in the v-myc and cmyc sequences also suggests that the nucleotide sequences of the two genes are closely related. For example, a SacI and a SalI site are located at similar positions in the two genes. It is also likely that the viral and cellular DNAs contain a ClaI site in analogous positions. The ClaI site in v-myc is located ca. 50 nucleotides to the 3' side of the SalI site that is shared by viral and cellular myc; the ClaI site in c-myc would probably be in the same position if the intron were removed from the cellular form of the gene.

How was c-myc acquired by a retrovirus? The results of our analysis of c-myc raise again the problem of how retroviruses might transduce cellular genes. The presence of one or more introns in c-myc precludes a simple DNA-DNA recombinational event; however, it is conceivable that intervening sequences could be excised from a transducing viral genome as a result of post-transcriptional processing. A prevalent model invokes fusion between the provirus of the transducing retrovirus and a cellular gene, followed by the genesis of a hybrid RNA from the fused transcriptional unit, encapsidation of the hybrid RNA in the virions of a competent retrovirus, and recombination between the RNA of the competent virus and the hybrid RNA (see reference 8 for an example of this sequence of events). It may be possible to test this model by joining suitable fragments of cloned retrovirus DNA to cloned c-myc DNA in vitro. Transfection of the chimeric DNA in the company of a competent helper virus might generate a defective retrovirus genome containing *myc* without introns.

J. VIROL.

What are the roles of the viral and cellular mycgenes in oncogenesis? There is reasonably extensive evidence for the involvement of the viral myc gene in the induction of neoplastic transformation. However, functional domains in v-mycthat are responsible for the pleiotropic oncogenic effects of the gene have yet to be identified and characterized. Mutants of MC29 with partial deletions in the myc gene have lost the ability to transform hematopoetic cells in vitro (19) and do not induce leukemias in infected birds (M. Hayman, personal communication). A comparison of the mutant viral myc genes with the two exons in c-myc might help to identify functional domains in v-myc.

Recent evidence indicates that augmented expression of c-myc may participate in the genesis of bursal tumors consequent to infection by avian leukosis virus (11). The exact role of cmyc in oncogenesis remains uncertain, however. For example, although DNA isolated from bursal tumors induced by avian leukosis virus can transform mouse fibroblasts in culture (3), c-myc is not responsible for the transformation (4). The availability of molecular clones of c-myc should facilitate evaluation of the gene's oncogenic and transforming potential.

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