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Amplification and Rearrangement of c-*myc* in Radiation-induced Murine Osteosarcomas[1]

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ABSTRACT

Fifty-one radiation-induced murine osteosarcomas were investigated for alterations in c-*myc* gene structure and c-*myc* expression. Amplification of c-*myc* was found in 30% of BALB/c tumors and 13% of NMRI tumors. A region of common proviral integration, *Mlvi-1*, localized on the same region on chromosome 15, was amplified concomitantly. Multiple copies of both loci were localized on double minutes. Three of the tumors with c-*myc* amplification also showed rearrangements of the c-*myc* gene region. One of these rearrangements included the 5′ and 3′-flanking sequences and the noncoding part of the third exon. Repetitive sequences were found in the 5′ region of the c-*myc* gene, and the 3′ flanking region was substituted by sequences normally present in a more distant part of chromosome 15. Increased levels of c-*myc* transcripts of apparently normal size were found in tumors carrying amplified c-*myc* sequences. Abnormally high expression of c-*myc* in some tumors was correlated with an early stage of osteogenic differentiation, suggesting the involvement of the c-*myc* gene in the control of the osteogenic differentiation of transformed cells.

INTRODUCTION

Osteosarcomas occur with a low natural incidence in both humans (1) and mice (2). An increased incidence of osteosarcomas has been observed in humans after radium therapy (1). The tumor can be induced experimentally in mice by bone-seeking radionuclides (2, 3). The mechanism of radiation induction of tumors, especially of osteosarcomas, is still poorly understood. Altered expression of the protooncogenes c-*sis* and c-*myc* has been observed in osteosarcoma cells (4–8). Amplification of the c-*myc* oncogene has also been demonstrated occasionally in virus-induced (7), radiation-induced, and spontaneous osteogenic sarcomas and/or cell lines derived from them (4, 9–11). The participation of other oncogenes, such as *fos* and *mos*, in osteosarcomagenesis is indicated by the fact that osteogenic tumors can be induced by the Finkel-Biskis-Reilly (FBR) murine osteosarcoma virus (12) and by the bone-adapted Moloney murine sarcoma virus (13). FBR and Moloney murine sarcoma viruses have acquired *fos* and *mos* sequences, respectively, which are structurally altered as compared with the normal cellular protooncogenes c-*fos* and c-*mos* (14–16). The involvement of the c-*fos* gene in abnormal osteogenesis in *fos*-transgenic mice has recently been reported (17).

In an attempt to gain further insight into the role of cellular protooncogenes in radiation-induced osteosarcomagenesis, we searched for structural changes in the protooncogenes c-*myc*, c-*sis*, c-*fos* and c-*mos*. We present here the first comprehensive study of alterations in copy number, structure, and expression of the c-*myc* oncogene in radiation-induced murine osteosarcomas. We observed amplification of a DNA region from the mouse chromosome 15 which includes the protooncogene c-*myc* as well as the proviral integration site *Mlvi-1* (18). This region was amplified in 30% of the BALB/c osteosarcomas and 13% of the NMRI tumors. In one tumor (TV) analyzed in detail, the flanking regions of the c-*myc* gene including the third exon had undergone substantial genomic rearrangements. The amplified region in cells from this tumor was located on extrachromosomal DMs.[3] The results suggest that the c-*myc* gene is involved in the multistep process of osteosarcomagenesis and the differentiation process of osteogenic cells.

MATERIALS AND METHODS

Animals and Tumors. Fifty-seven osteosarcomas were isolated from female mice of 4 different strains. Fifty-three osteosarcomas (22 tumors from NMRI, 29 from BALB/c, and 2 from C3H×101/F_1 mice) were induced by i.p. injection of the bone-seeking radionuclides ^{224}Ra, ^{227}Th, ^{177}Lu, and ^{227}Ac as described (2). One tumor was induced by Finkel-Biskis-Jinkins (FBJ) murine sarcoma virus in a C3H mouse, and three tumors arose spontaneously, of which OTS79 (BALB/c) is shown in the results. Tissue cell cultures were established from 3 of the 57 primary tumors prior to this investigation (6, 19). Forty-nine primary tumors were transplanted once before analysis, as described previously (20). Five osteosarcomas were established as transplantation lines, maintained, and passaged every fourth wk. The transplantation line TV, induced in an NMRI mouse by ^{224}Ra in 1973, was examined in more detail. DNA from tumors transplanted between 1974 and 1985 was analyzed. The osteosarcoma TV was also established as a cell line, as described previously (6).

Cytogenetic Analysis. A tissue cell culture from the osteosarcoma TV386/1 was used for cytogenetic investigation of tumor cells. Standard cytogenetic techniques were applied for GTG, QFQ, and CBG banding. Staining with the AT-specific fluorochrome DAPI was performed according to the method of Lin *et al.* (21).

For the chromosomal localization of the I10 sequence, embryo fibroblasts were grown from a strain of mice which carries, as the only metacentric chromosome, the marker of chromosome Rb(4.5)4Rma in a homozygous form (22). In the karyotype, chromosome 15 is easily recognized as the short arm of this single pair of metacentric chromosomes. The chromosomes were prepared according to standard techniques (22).

In situ hybridizations were carried out as described previously in detail (22). Mouse satellite DNA was used for *in situ* hybridization to c-heterochromatin. The fragments were prepared from Rb(4.15)4Rma DNA that had been completely digested with the endonuclease *Ava*II. The DNA fragments were separated by agarose gel electrophoresis, and the 240-base pair satellite DNA was isolated (23) and directly used for nick translation.

The probes were labeled with [^{3}H]dCTP and [^{3}H]dTTP to a specific activity of 2 to 5×10^4 dpm/μg of DNA. When using single copy DNA probes, the slides were exposed for 2 and 4 wk. The slides were exposed for 1 wk after hybridization to satellite DNA.

DNA Preparation and Analysis. High-molecular-weight DNA from osteosarcomas and normal tissues was prepared as described previously (20). After electrophoresis in 0.8 to 1.0% agarose gels, the separated fragments were transferred onto nitrocellulose filters. The hybridization and washing were performed under stringent conditions for murine

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[3] The abbreviations used are: DM, double minute; DAPI, 4′,6′-diamidino-2-phenylindole; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; HSR, homogeneously staining region.

probes and the human probe pλHAc-69A (hybridization, 50% formamide:5 × SSC:5 × Denhardt's solution:0.02 M phosphate buffer: 0.05 mg/ml of carrier DNA at 42°C; last wash, 0.1 × SSC:0.1% SDS at 65°C) and less stringent conditions for all other probes (40% formamide:6 × SSC:5 × Denhardt's solution:0.02 M phosphate buffer:0.05 mg/ml of carrier DNA at 35°C and 1 × SSC:0.1% SDS at 65°C).

DNA Hybridization Probes. Inserts of the following clones were used for hybridizations: C-*myc*: pSVc-myc1 (24), a murine 4.8-kilobase *Xba*I/*Bam*HI fragment, containing the second and third exons and 3′-flanking sequences; U157/11 (25), a human 1.3-kilobase *Cla*I/*Eco*RI fragment, homologous to the third exon and 3′-flanking sequences; S421/1 (26), a human 0.9-kilobase *Pvu*II fragment, hybridizing to the promoters and the first exon; *V-sis*: pC60 (27), a 0.9-kilobase *Pst*I/*Xba*I fragment; *V-mos*: psrc (16), a 1.2-kilobase *Xba*I/*Hin*dIII fragment; *Mlvi-1*: pTS25E/P (28), a 1.3-kilobase *Eco*RI/*Pvu*II fragment from rat; for *in situ* hybridization: pTS26P/P (18, 29), a 0.6-kilobase *Pvu*II fragment from rat; *Mlvi-2*: pTS10 (30), a 1.8-kilobase *Hin*dIII fragment from rat; *Hba/3ps*: α-ψ3 (31), a murine 4.3-kilobase *Eco*RI fragment; virus integration site: pOTS25E/P (20), a murine 0.5-kilobase *Eco*RI/*Pst*I fragment; pseudoactin: pλHAc-69A (32), a human 3.6-kilobase *Hin*dIII fragment; osteopontin: pBS$^+$-ROP (33), a 1.2-kilobase *Eco*RI fragment from rat.

Cloning. A partial library was established from genomic DNA from the tumor TV389/1 as described previously (34). Briefly, 250 μg of high-moleclar-weight DNA were digested to completion with the restriction endonuclease *Eco*RI. DNA fragments from 18 to 30 kilobases, which contained the c-*myc* sequences, were enriched by electroelution from a preparative agarose gel. The *Eco*RI fragments were cloned into the bacteriophage EMBL3A (35) and screened with the murine c-*myc* probe pSVc-myc1. The DNA from 14 positive clones was isolated by a rapid small-scale bacteriophage DNA preparation (34). DNA was isolated by a large-scale preparation of recombinant bacteriophage λ from two clones (36). For insert preparation, the 5′ arms of the recombinant phages were digested with the endonucleases *Nru*I and *Eco*RI. The complete *Eco*RI *myc* insert was separated by gel electrophoresis and used as a hybridization probe.

For subcloning, recombinant EMBL3A bacteriophages were digested to completion with *Bam*HI. The fragments were ligated into the plasmid pUC19 (37). The DNA of recombinant plasmids was prepared by a rapid boiling method (34) and directly used for DNA analysis and nick translation.

The murine c-*myc* sequences from the EMBL/Gen Bank data base, VAX PIR-Program, release 12 (European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany; 38, 39), were used for comparison of the restriction maps.

RNA Preparation and Analysis. Total RNA isolation, RNA slot blots, RNA Northern blots, and scanning densitometer tracing were carried out as described previously (20).

RESULTS

Amplification of c-*myc*. The structure of the c-*myc* protooncogene was examined in the DNA of 57 murine osteosarcomas, of which 53 were radiation induced. Amplification of c-*myc* was found in 9 of 30 tumors from BALB/c mice and in 3 of 23 osteosarcomas from the NMRI strain. No altered c-*myc* structure was observed in 4 osteosarcomas from 2 other mouse strains. All 12 osteosarcomas containing amplified c-*myc* sequences were radiation induced. A selection of these tumors is shown in Fig. 1. Digestion of the DNA from these 12 osteosarcomas with the restriction endonuclease *Eco*RI generated multiple c-*myc*-containing fragments with the normal size of 20 kilobases (40) (Fig. 1, *I* and *II*). The tumor OTS60 showed an additional rearranged *Eco*RI fragment. Densitometer tracing measurements showed the *Eco*RI fragments to be amplified up to 40-fold (Table 1).

We compared the extent of the amplification of c-*myc* DNA sequences with the level of c-*myc* RNA transcripts in 10 selected

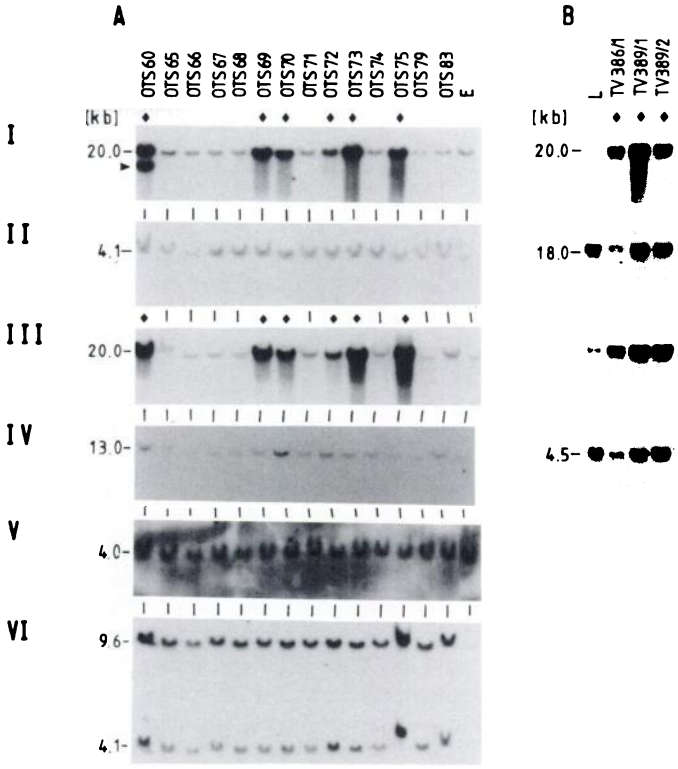

Fig. 1. Coamplification of c-*myc* and *Mlvi-1*. Southern blot analysis of tumor DNA. In *A*, 10 μg of high-molecular-weight DNA from 14 primary tumor transplants and a 19-day-old embryo (*E*) from BALB/c mice were digested with *Eco*RI (*I* and *II*); *Kpn*I (*III* and *IV*); *Sac*I (*V*), and *Bgl*I (*VI*). In *B*, 10 μg of high-molecular-weight DNA from normal NMRI liver (*L*) and three tumors of the transplantation line TV (NMRI) were digested with *Eco*RI (*I* to *IV*). After agarose gel electrophoresis, the DNA was transferred onto nitrocellulose filters and hybridized to pSVc-myc1 (*I*); probes for a single copy gene pOTS25E/P (*II*) in *A* and v-*mos* in *B*; *Mlvi-1* (*III*); *Mlvi-2* (*IV*); v-*sis* (*V*); and α-globin pseudogene Hba-3ps (*VI*). In *B*, the same filter was hybridized successively to each probe. For pSVc-myc1 the hybridization was exposed for 2 h. The hybridization signal of the normal tissue was detectable after 1-day exposure. Tumors containing amplified c-*myc* and *Mlvi-1* sequences (◆) and rearranged DNA fragments (▶) are indicated.

Table 1 *Amplification and expression of c-myc sequences in ten murine osteosarcomas compared with normal BALB/c liver*

Tissue	c-*myc* amplification (fold)[a]	c-*myc* expression (fold)[b]
BALB/c liver	1 ×	1 ×
TV 389/1	40×	50×
OTS-60	5×	7×
OTS-69	15×	6×
OTS-70	20×	6×
OTS-73	28×	12×
OTS-75	38×	50×
OTS-78	1×	1×
OTS-79	1×	2×
OTS-82	1×	2×
OTS-83	1×	2×

[a] Determined by scanning densitometer tracing of autoradiograms of Southern blots.

[b] Determined by scanning densitometer tracing of autoradiograms of slot-blots. The signal obtained with liver RNA is given as value 1.

osteosarcomas, of which 6 carried an amplified c-*myc* region (Table 1). The expression of c-*myc* RNA was increased 6- to 50-fold in those tumors which harbored multiple gene copies of c-*myc*. The amount of c-*myc* RNA roughly correlated to the copy-numbers of the amplified c-*myc* genes. One tumor, OTS81, did not show an increased expression of c-*myc*, although the gene was amplified in the tumor (data not shown). The tumors which contained only a single copy of c-*myc* expressed RNA at a low level comparable to that in the liver of a normal BALB/c mouse.

To identify the extent of the amplified region in murine osteosarcomas, tumor DNA was hybridized to probes from four other loci also located on chromosome 15. The alignment of the loci from proximal to distal was previously established as *Mlvi-2, Hba-3ps, Mlvi-1*, c-*myc* and c-*sis* (22, 41). The *Mlvi-1* locus was amplified in all tumors containing multiple copies of c-*myc* sequences, but not in any of the tumors without c-*myc* amplification (Fig. 1*III*). *Mlvi-1* sequences were amplified to a higher degree than c-*myc* in the tumors OTS35/1 and OTS95/1 from NMRI mice (data not shown). The α-globin pseudogene *Hba-3ps*, localized in close proximity to *Mlvi-1* (41), was present with a normal copy number in all tumors, as were *Mlvi-2*, located closer to the centromere, and the protooncogene c-*sis*, distal to c-*myc* (Fig. 1). In addition, we analyzed the genomic structure of the protooncogenes c-*fos* and c-*mos*, as well as N-*myc*, which is also often amplified in tumor DNA (42). In contrast to c-*myc*, these protooncogenes appeared to be normal in their copy number in all osteosarcomas and there was no evidence of gross rearrangements of these genes.

Localization of the Amplified c-*myc* and *Mlvi-1* Sequences on Double Minutes. Cytogenetic investigation of the tumor TV386/1 revealed a great variety of chromosomal aberrations in 33 examined metaphases. The karyotype varied from 48 to 56 chromosomes per metaphase. Dicentric chromosomes, DMs, a HSR and different marker chromosomes were observed (Fig. 2*A*).

Fig. 2*B* shows the identification of amplified c-heterochromatin in the HSR with QFQ banding, DAPI staining, CBG banding, and *in situ* hybridization to murine satellite DNA. The satellite DNA was arranged in a manner which suggests 3-fold amplification of a large unit. The chromosome carrying the HSR could not be identified.

The number of DMs was variable between different cells (Fig. 2*C*). Some cells contained more than 100 DMs. *In situ* hybridization to the c-*myc* probe revealed strong hybridization signals on DMs in tumor cells from TV386/1 (Fig. 2*CII*). Similar results were obtained using a probe for *Mlvi-1*, although the hybridization signal was less intense (data not shown). The probe for *Hba-3ps*, localized in close proximity to *Mlvi-1* on chromosome 15 (41), revealed no hybridization to DMs (data not shown).

Rearrangement of c-*myc* in Murine Osteosarcomas. Apparently normal sized restriction fragments of the amplified c-*myc* protooncogene were observed in most radiation-induced osteosarcomas. However, after digestion of the tumor DNA with *Bgl*I, three tumors (OTS35/1, OTS60, and the transplantation line TV) showed a rearrangement in the c-*myc* gene in addition to the amplification of the protooncogene. Following digestion with the restriction endonuclease *Eco*RI, rearrangement was only detected in OTS60 (Fig. 1*I*).

We selected the osteosarcoma transplantation line TV for more detailed investigation of the rearrangement of c-*myc* genes. These tumors showed high c-*myc* amplification, and cell lines derived therefrom were available for the cytogenetic investigations discussed above. The restriction endonuclease patterns of the amplified *myc* sequences of this transplantation line were stable over 11 yr (Fig. 3). DNA from the osteosarcoma TV389/1 (Fig. 1*B*) was used to clone the c-*myc*-containing *Eco*RI fragments.

We isolated 14 clones of recombinant phages (7 clones containing a 19.6-kilobase *Eco*RI fragment, designated as type I, and 7 clones containing a 20.2-kilobase fragment, designated as type II) from a partial library of the genome from the tumor TV389/1 (Fig. 4*A*). All 14 clones contained an identical 7.8-kilobase *Bam*HI fragment which hybridized to probes representing the murine second and third exon (pSVc-myc1) and the human third exon (U157/11).

Restriction mapping of one *Eco*RI fragment of type I and one of type II revealed the same rearranged *myc* gene (designated rc-*myc*) in both types (Fig. 4*C*). The restriction map of the cloned rc-*myc* gene between the *myc* promoters and the third exon was indistinguishable from the restriction map of the germline c-*myc*. However, different restriction endonuclease sites at the 3′ side of the third exon indicated a rearrangement in this region. The rearrangement included the cloned 3′-flanking sequence of rc-*myc* up to the *Eco*RI cloning site (Fig. 4*A*). This 3′-flanking sequence of rc-*myc* of at least 6.9 kilobases differed from germline DNA in restriction endonuclease sites in both *Eco*RI fragments, type I and type II. The breakpoint in the rc-*myc* gene is located between the *Xho*I site and the *Hin*dIII site in the third exon (Fig. 4*C*). Only the 5′ fragment of this *Xho*I site and 5′ of the *Hin*dIII site in the third exon hybridized to the probes pSVc-myc1 and U157/11. Sequence data (38, 39) indicate that the rearrangement occurred downstream of the coding region. All 14 cloned *Eco*RI fragments, either type I or type II from the tumor TV389/1, were identical in the rearranged 3′ region.

The 6.1-kilobase 5′-flanking sequence next to the rc-*myc* gene seemed to be normal in both type I and type II *Eco*RI fragments, when compared with the germline map of Corcoran *et al.* (40) (Fig. 4*A*). There was no evidence for major deletions, insertions, or inversions in this region. The 5′ end of the clone type I showed a restriction map similar to that of the germline map. However, the most distal 5′ part of the type II *Eco*RI fragment contained different restriction endonuclease sites than the same area in the type I fragment (Fig. 4*B*). The type-specific 5′ sequence of clone type I, contained within a 2.2-kilobase *Eco*RI/*Bgl*II fragment, and that of clone type II, contained within a 2.7-kilobase *Eco*RI/*Bgl*II fragment, did not cross-hybridize.

In summary, all 14 clones contained the same rc-*myc* gene including 5′- and 3′-flanking sequences. Type I and type II *Eco*RI fragments, however, differed in their most distal 5′ sequence. The restriction map of the 3′ flanking sequence of rc-*myc* was different from that of the normal c-*myc* sequence.

Genomic analysis of the osteosarcoma TV confirmed the structural changes in the cloned rc-*myc* region (data not shown). Only normal sized, amplified fragments hybridized to the probes for the first exon (S421/1), the second exon, and the first part of the third exon (pSVc-myc1). Hybridization probes pSVc-myc1 and U157/11 only revealed rearranged fragments for the third exon and 3′ flanking sequences. Strongly hybridizing, amplified fragments were rearranged and had the same size as those of the cloned rc-*myc* gene. In addition, weakly hybridizing rearranged fragments of different sizes indicate further rearrangements in the c-*myc* gene from the tumor TV.

The 3′-flanking foreign region of rc-*myc* was also strongly amplified in the genome of the tumor TV (Fig. 5*AI*). Two rearranged *Eco*RI fragments of 20 and 13.9 kilobases were detected in addition to the normal fragment of 10.4 kilobases after hybridization to a 2.3-kilobase 3′ *Bam*HI/*Eco*RI fragment (subclone I10) as probe. In contrast to the 20-kilobase fragment, the fragments of 10.4 and 13.9 kilobases did not hybridize to any of the three *myc* exons (S421/1 and pSVc-myc1 in Fig. 1*B*). In one additional osteosarcoma, OTS73, the I10 sequence was also amplified, and the *Eco*RI fragment was rearranged (Fig. 5, *AI* and *BI*).

The chromosomal localization of the I10 sequence in murine

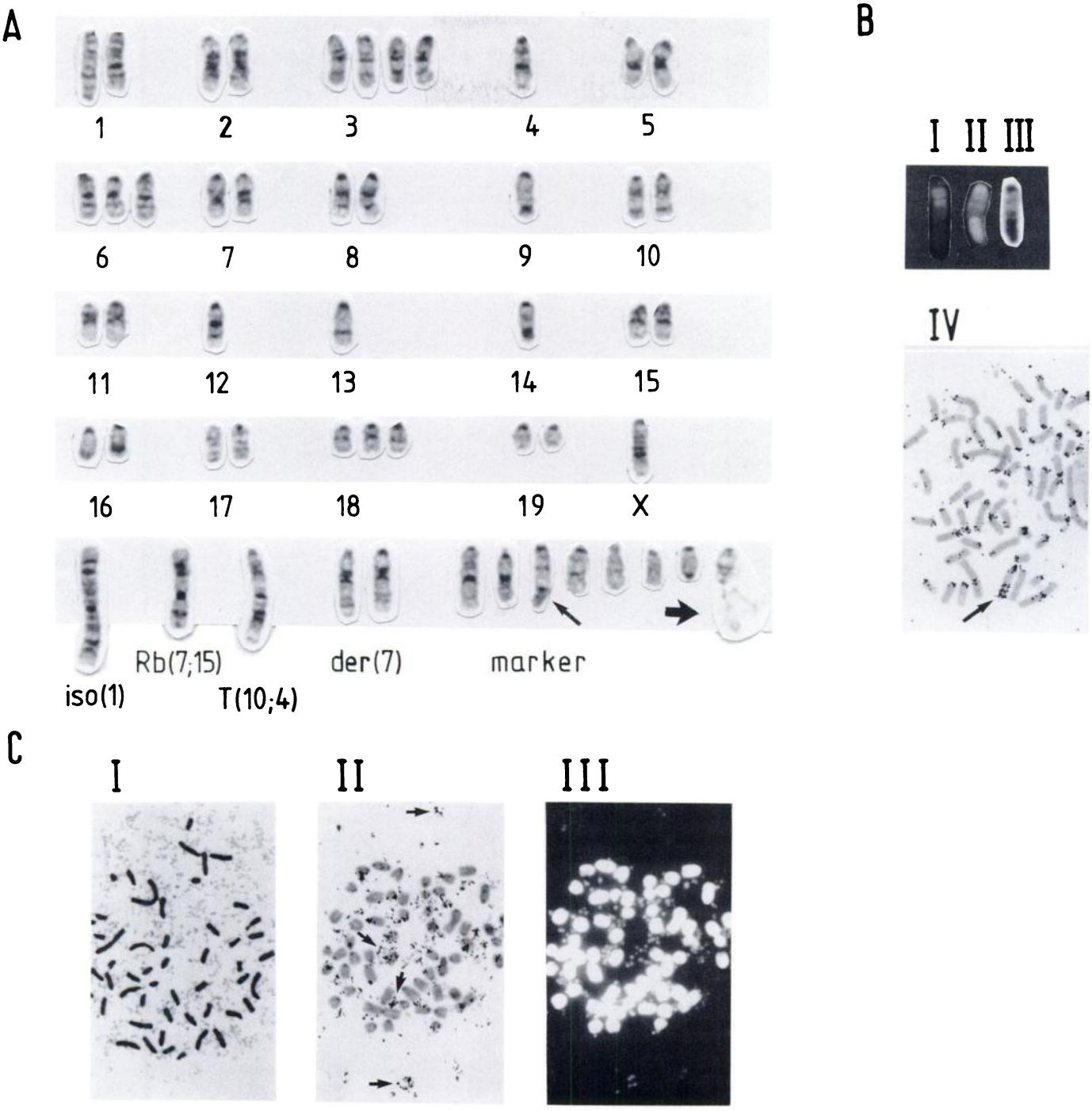

Fig. 2. Cytogenetics of the tumor TV386/1. *A*, GTG-banded karyotype of tissue culture cells from TV386/1. A number of aberrant chromosomes were observed in 33 metaphases. Some were identified as iso(1), iso(19), Rb(7.15), der(7), T(7;?), and T(10;4) chromosomes. The marker chromosomes in this metaphase are shown at the *bottom*. In this cell, DMs were found to be attached to one of the marker chromosomes (*large arrow*). A HSR is indicated by a *small arrow*. *B*, cytogenetic analysis of the HSR in cells from the tumor TV386/1: *I*, QFQ banding of an HSR-containing chromosome; *II*, positive DAPI staining; and *III*, CBG banding of the same chromosome. *IV, in situ* hybridization to satellite DNA. The hybridization to the HSR on the same chromosome in this metaphase is indicated by a *small arrow*. *C*, cytogenetic analysis of DMs. *I*, a Giemsa-stained metaphase with more than 100 DMs, *II, in situ* hybridization of a Giemsa-stained metaphase to pSVc-myc1; *III*, the same metaphase as in *II*, QFQ stained and overilluminated to visualize DMs. The *arrows* point to hybridization signals in *II*, overlaying the DMs as shown in *III*.

germline DNA was determined by *in situ* hybridization (Fig. 5*C*). The I10 sequence was found to be located on chromosome 15 at a position indistinguishable from the c-*myc* locus in the region 15D2/3 (22).

The type-specific 5′ sequence of clone type II was strongly amplified in the genome of the osteosarcoma TV (Fig. 5*AII*). Hybridization of the 5′ *Eco*RI/*Bam*HI fragment of 0.8 kilobases (subclone II66) to germline DNA resulted in a smear rather than discrete bands, indicating the presence of repetitive elements. However, the probe II66 revealed an amplified *Bam*HI fragment of 5.5 kilobases in all osteosarcomas carrying amplified c-*myc* genes (Fig. 5, *AII* and *BII*). No specific bands were detected in other tumors or normal tissue.

Correlation of High c-*myc* Expression with Low Expression of Osteopontin. Although c-*myc* was rearranged in some tumors, all of these tumors expressed c-*myc* transcripts of normal size (Fig. 6*I*). Two tumors showed an unusual enhanced RNA expression. The increased c-*myc* mRNA level in the tumors

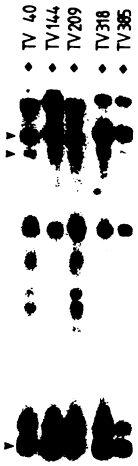

Fig. 3. Genomic rearrangement of the amplified c-*myc* protooncogene in tumor DNA. Ten μg of high-molecular-weight DNA from five tumor transplants of the osteosarcoma TV were digested with *Bgl*I. After Southern blotting, the filter was hybridized to pSVc-myc1. TV40 was transplanted in 1974 and TV385 in 1985. Tumors containing amplified c-*myc* genes (◆) and rearranged DNA fragments (▶) are indicated.

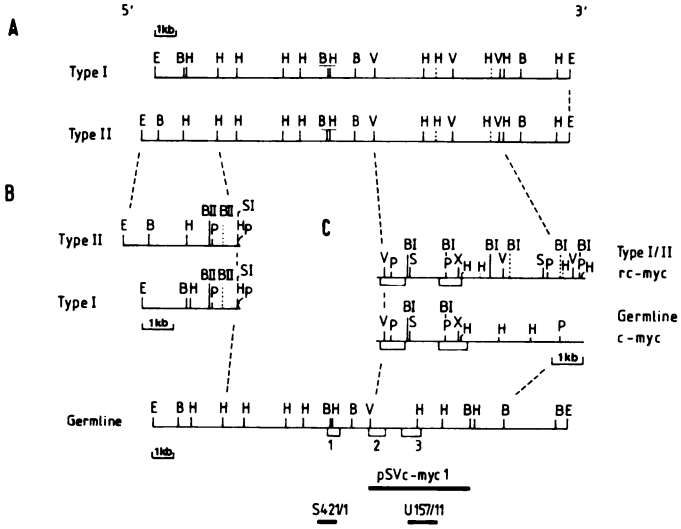

Fig. 4. Restriction maps of the cloned rc-*myc* sequences from the osteosarcoma TV389/1. *A*, rc-*myc* containing *Eco*RI fragments of types I and II from the tumor TV389/1 (NMRI mouse); *B*, type-specific 5′ sequences of the *Eco*RI fragments of types I and II; *C*, second and third exons of the germline c-*myc* gene from the NMRI strain and the rearranged rc-*myc* from the osteosarcoma TV389/1. The restriction map of the *Eco*RI fragment from the murine germline DNA (40; AKR mouse) is scaled down to 19.6 kilobases. *Open boxes* indicate the exons of c-*myc* and rc-*myc; bars below the map* indicate three different c-*myc* hybridization probes S421/1, pSVc-myc1, and U157/11. Restriction endonuclease site abbreviations: *B, Bam*HI; *BI, Bgl*I; *BII, Bgl*II; *E, Eco*RI; *H, Hin*dIII; *P, Pvu*II; *S, Sac*I; *SI, Sal*I; *V, Eco*RV; *X, Xho*I.

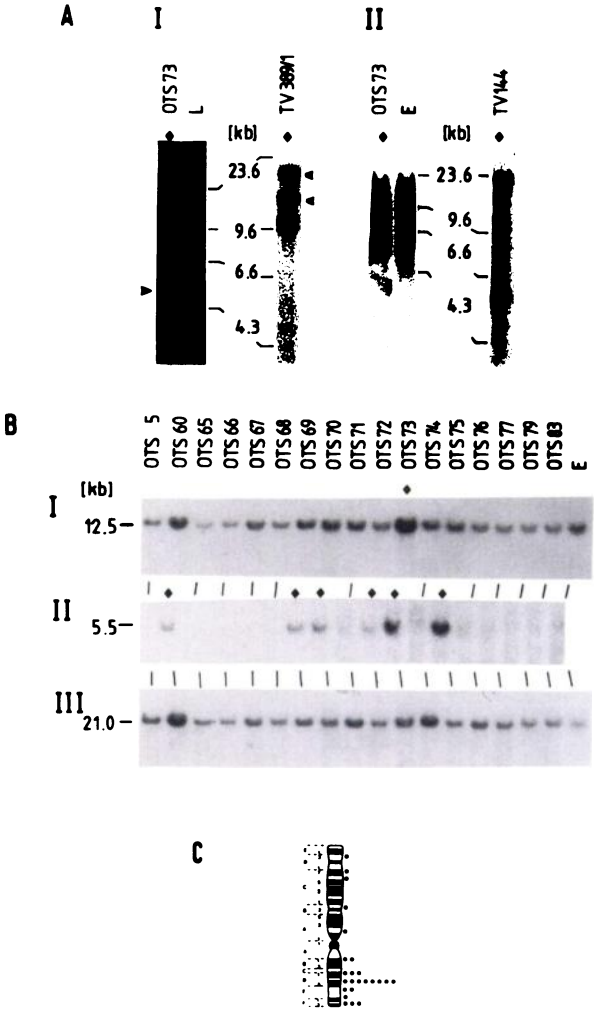

Fig. 5. Southern blot analysis of 3′ and 5′ sequence of rc-*myc* in the genomes of the osteosarcoma transplantation line TV (NMRI) and of 17 osteosarcomas from the BALB/c strain. *A*, Southern blot analysis of DNA from liver (*L*), embryo (*E*), and from the tumors OTS73, TV389/1, and TV144 digested with *Eco*RI. The ethidium-bromide-stained gel showed bands of satellite DNA, indicating complete digestion of genomic DNA. The DNA was hybridized to subclone I10, a 2.3-kilobase *Bam*HI/*Eco*RI 3′ fragment from rc-*myc* (*I*) and to subclone II66, a 0.8-kilobase *Eco*RI/*Bam*HI 5′ fragment from rc-*myc* (*II*). *B*, Southern blot analysis of tumor DNA from the BALB/c strain as shown in Fig. 1*A*. DNA of primary tumor transplants and of a BALB/c embryo (*E*) was digested with *Bam*HI. The DNA was hybridized to subclone I10 (*I*), to subclone II66 (*II*), and to pOTS25E/P (*III*), a probe for a murine single copy locus. Tumors containing multiple sequence copies (◆) and rearranged DNA fragments (▶) are indicated. *C*, localization of I10 sequences on chromosome 15 by *in situ* hybridization. Schematic presentation of grain distribution on marker chromosome Rb(4.15)4Rma in which the murine chromosome 15 is attached to chromosome 4. Twenty-eight metaphases were evaluated with a total number of 87 grains. Nineteen grains were localized on Rb(4.15)4Rma, and 11 grains at the specific region 15D2/3.

TV389/1 and OTS75 was accompanied by a low expression of osteopontin (bone sialoprotein), a marker for osteogenic cells (Fig. 6*II*) (33, 43). The expression of bone gla protein, another marker of highly differentiated osteogenic cells (44), was also reduced (data not shown).

DISCUSSION

Altered expression of the cellular protooncogenes c-*myc* and c-*sis* in osteosarcoma cells has been reported previously (4–8). Here we investigated the structure of the oncogenes c-*myc*, N-*myc*, c-*sis*, c-*mos*, and c-*fos* in 57 murine osteosarcomas, of which 53 were radiation induced. We found no indication for genomic alterations of the protooncogenes N-*myc*, c-*sis*, c-*mos*, and c-*fos*. These genes were apparently normal in their genomic copy number and revealed no gross rearrangements. However, 30% of the BALB/c tumors and 13% of the NMRI osteosarcomas, all radiation induced, contained an amplified c-*myc* gene. Because DNA from the small-sized primary tumors was not available in sufficient amount, tumor material was expanded by transplantation into isogeneic mice. We have reported previously that several transplants originating from the same primary tumor show the same pattern of somatically acquired proviruses in the genome (20). This indicates the expansion of a population of cells which already preexisted in the primary tumor in a considerable portion of the tumor mass. Similarly we have observed the same state of c-*myc* amplification in parallel in transplants from 10 of 11 investigated primary

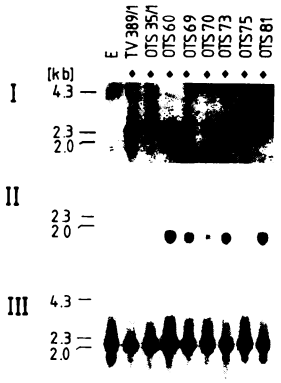

Fig. 6. c-*myc* and osteopontin transcripts from radiation-induced osteosarcomas. Ten μg of total RNA from a 19-day-old embryo (*E*) and from eight osteosarcomas were transferred onto a Biodyne A nylon membrane after agarose gel electrophoresis. The filters were hybridized to pSVc-myc1 (*I*), to osteopontin (*II*), and to actin (*III*), successively. The tumors containing amplified c-*myc* genes (◆) are indicated.

tumors; *i.e.*, either c-*myc* was amplified in all transplants from the same primary tumor or in none of them.[4] This strongly suggests that the c-*myc* amplification had occurred in primary tumors prior to transplantation. It was not possible, however, to determine whether c-*myc* was amplified at an early or a later stage in the development of the primary tumors.

The c-*myc* copy number and the c-*myc* RNA level roughly correlated. Tumors which showed the highest c-*myc* RNA levels (TV, OTS75) also contained the highest c-*myc* copy numbers. However, the gene dosage was not accurately reflected in the transcript levels, and one tumor carrying a c-*myc* amplification did not reveal an enhanced level of c-*myc* RNA.

It has been shown recently for several tumors that an amplicon often includes a large DNA region (45, 46). To study the extent of the amplified region in the osteosarcoma DNA, we used hybridization probes for four additional loci on the murine chromosome 15: *Mlvi-2*; *Hba-3ps*; *Mlvi-1*; and c-*sis* (29, 30, 41, 47). The locus *Mlvi-1* was coamplified in those tumors carrying multiple copies of c-*myc*, whereas the loci *Mlvi-2, Hba-3ps*, and c-*sis* showed a normal copy number in all osteosarcomas. In the tumors carrying the amplified region, one end of the amplicon may be indicated by the nonamplified locus *Hba-3ps*, which has been localized in close proximity to *Mlvi-1* by *in situ* hybridization (41). The absolute extent of the amplicon distal to c-*myc* was not determined, but c-*sis* which maps to 15E (22) was not amplified in the genome of the osteosarcomas.

Only a limited amount of karyotype data exists for cells derived from osteosarcomas (7, 19). Cytogenetic investigation of the osteosarcoma TV revealed a variable karyotype with extrachromosomal DMs, a HSR and different marker chromosomes. DMs and HSRs are chromatin structures which contain amplified sequences in tumor cells and are considered to be alternative cytological manifestations of the same DNA sequences (7, 42, 48). *In situ* hybridization localized c-*myc* and *Mlvi-1* sequences exclusively to extrachromosomal DMs, and not to HSRs, in cells from the osteosarcoma TV. The HSR contained amplified satellite DNA.

The amplified c-*myc* gene was rearranged in three osteosarcomas. One tumor from the osteosarcoma transplantation line TV was selected for detailed analysis of the rearrangement in the c-*myc* gene. Two types of clones were isolated from a partial genomic library derived from the tumor TV389/1. Restriction mapping identified a rearrangement of the c-*myc* gene (rc-*myc*) common to both types of clones. The breakpoint was located 3′ of the *Xho*I site, near the *Hin*dIII site in the third exon, and was recombined with a sequence which was not related to the normal 3′-flanking region. According to sequence data (38, 39), the translational stop codon is located 5′ of this *Xho*I site in the third exon. Thus the coding region of rc-*myc* does not seem to be rearranged. The polyadenylate addition site is located 180 base pairs 3′ of the *Hin*dIII site in the third exon (38, 39). This indicates that nontranslated 3′ sequences in the third exon of rc-*myc* were probably removed, either by deletion, inversion, or insertion of a foreign DNA sequence. The 3′ nontranslated sequence of several genes, including c-*myc*, has been reported to be essential for the stability of their mRNA (49, 50). In the c-*myc* gene, a sequence of 140 base pairs in the 3′ nontranslated region appears to be primarily responsible for the short half-life of its mature mRNA, due to posttranscriptional control mechanisms (51–53). A human plasma cell myeloma has recently been demonstrated to carry a c-*myc* gene with a rearranged 3′ nontranslated sequence, which shows an increased mRNA half-life (54). There are no data available to determine whether the increased level of c-*myc* mRNA in the tumor TV originated from a normal or a rearranged amplified gene. However, all strongly amplified rearranged *myc* fragments corresponded in size to the restriction endonuclease fragments of the cloned rc-*myc* gene. The normal 3′ untranslated sequence of c-*myc* was replaced in the rc-*myc* gene by recombination with an unrelated sequence, which as a consequence might have altered the posttranscriptional control of the rc-*myc* mRNA by affecting its normally short half-life. In all osteosarcomas only *myc* mRNA transcripts of the normal size were observed, despite the rearrangements in the c-*myc* region in the tumors TV, OTS35/1, and OTS60.

The rearrangement of the protooncogene c-*myc* in the osteosarcoma TV must have occurred at a stage in the tumorigenesis preceding the major amplification process of this genomic region. All 14 isolated clones contained the identical rc-*myc*. Further, the rearranged sequence was amplified in the genome. A translocation of a c-*myc* allele in the osteosarcoma TV, as observed in B-cell lymphoma (42), is unlikely because we mapped the foreign 3′-flanking sequence to the region D2/3 on chromosome 15, close to the c-*myc* protooncogene (22). The unrelated sequence, rearranged with the c-*myc* gene in the tumor TV389/1, was amplified in one additional tumor, which also contained amplified c-*myc* fragments. Therefore, the unrelated 3′ sequence, which is rarely affected by the amplification process, apparently marks the end of the amplicon.

The rc-*myc* gene in the genome of the tumor TV389/1 appeared to be normal when compared with the restriction map of the germline c-*myc* from the promoters to the breakpoint in the third exon. The 5′-flanking sequence of 6.1 kilobases next to rc-*myc* in both type I and II *Eco*RI fragments was also indistinguishable from the germline c-*myc* recently mapped by Corcoran *et al.* (40). However, 5′ of this 6.1-kilobase sequence, both fragment types differed in their restriction endonuclease sites, and the novel DNA fragments did not cross-hybridize. The type II-specific region, cloned from the genome of TV389/1, was amplified in all the osteosarcomas also containing multiple copies of c-*myc*. Thus this cloned 5′ sequence is located within the amplicon which is often generated during osteosarcomagenesis. Most likely, this cloned type-specific region is composed of a unique sequence, giving rise to discrete bands in tumor DNA after its amplification, as well as repetitive elements, which generated a smear of hybridizing bands in the normal genomic DNA. Repetitive sequences were recently dem-

[4] Unpublished data.

onstrated to be involved in the amplification process, where they are apparently necessary for the recombination of the multiple copies generated (45). Fifty % of the clones carried the rearrangement of this 5′ sequence. Since all clones were identical in the rearrangement of the third exon, it is most likely that the 5′ rearrangement occurred as an independent second event at the beginning of the amplification process. The amplification of the whole region resulted in a limited number of different sized fragments including those carrying 3′ and type II-specific 5′-flanking sequences of rc-*myc*. A similar homogeneity of amplified DNA regions was recently shown in several neuroblastomas for N-*myc* amplicons (46).

Tumorigenesis is considered to be a multistep process (42, 55). Cytogenetic examination of the osteosarcoma TV revealed that multiple changes occurred in different tumor cells. During one of the steps in osteosarcomagenesis, a large region was amplified. Both the c-*myc* gene and the locus *Mlvi-1* are involved in the tumorigenesis of various neoplasias (18, 42). However, we cannot exclude the participation in osteosarcomagenesis of as yet unidentified genes which might be located on the affected region.

Osteosarcomas can be classified into three groups with respect to the involvement of the c-*myc* oncogene in the tumorigenic process. The first type of tumor has no apparent alteration of the gene structure and does not show abnormal expression of the c-*myc* gene. In this case, c-*myc* is probably not involved in tumorigenesis. The second group comprises tumors in which amplification or rearrangement of the c-*myc* gene caused an enhanced expression of mRNA. In these tumors the c-*myc* transcripts may stimulate the proliferation of the transformed cells. The third type of osteosarcoma showed a highly increased level of c-*myc* mRNA transcripts and a low level of osteogenic marker gene expression. Recently, it has been demonstrated that high constitutive c-*myc* expression results in a block of terminal differentiation (56). This is in good agreement with our observation that the osteogenic tumors expressing high levels of c-*myc* mRNA showed a dramatically decreased expression of the osteogenic markers osteopontin and bone gla protein. A similar observation has recently been made in a clonal cell line derived from a spontaneous murine osteosarcoma (6). This indicates that high c-*myc* expression in osteogenic tumors may determine a relatively early osteogenic differentiation level rather than cell proliferation. In this respect, the various groups of tumors may reflect different levels of malignancy in osteosarcomagenesis. Gene amplification alone may not be sufficient for the major effects observed in transformed osteogenic cells. Additional mutational events or DNA rearrangements may eventually lead to an overexpression of the c-*myc* gene which could then affect the osteogenic phenotype. These mechanisms could contribute to the known heterogeneity of osteogenic tumors and affect their pathogenicity.

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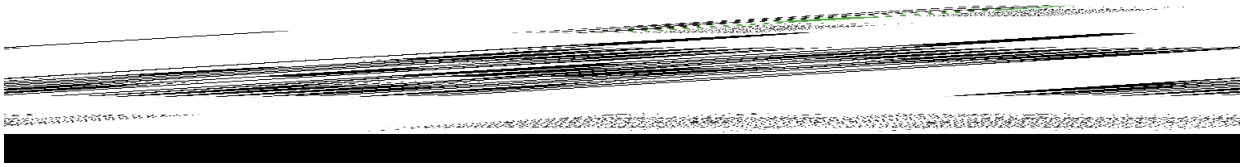

Amplification and Rearrangement of c-*myc* in Radiation-induced Murine Ostosarcomas

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