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Molecular Analysis and Chromosomal Mapping of Amplified Genes Isolated from a Transformed Mouse 3T3 Cell Line

Linda Cahilly-Snyder,[1] Teresa Yang-Feng,[2] Uta Francke,[2] and Donna L. George[1,3]

[1]*Department of Human Genetics and* [3]*Howard Hughes Medical Institute, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104; and* [2]*Department of Human Genetics, Yale University, School of Medicine, New Haven, Connecticut 06510*

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Abstract—*We are exploring the origin and function of amplified DNA sequences associated with double minutes (DMs) in a spontaneously transformed derivative of mouse 3T3 cells. Toward that goal, we have constructed a cDNA library using RNA from these cells and have isolated cDNA clones representing sequences that are amplified and overexpressed in these 3T3-DM cells. From results of Northern- and Southern-blot analyses, we conclude that these cDNAs represent two distinct genes, which we have designated* mdm-1 *and* mdm-2. *Using DNAs from a panel of Chinese hamster–mouse somatic cell hybrids together with in situ hybridization protocols for gene mapping studies, we have found that these DM-associated, amplified DNA sequences originate from mouse chromosome 10, region C1-C3. Sequences homologous to* mdm-1 *and* mdm-2 *are present in the genomes of several species examined, including that of man.*

INTRODUCTION

There is increasing evidence that the altered or inappropriate expression of specific cellular genes, termed oncogenes, may play a role in the initiation and/or progression of tumorigenesis (for review, see references 1, 2). One genetic mechanism which results in overexpression of such genes is gene amplification. The presence of amplified DNA sequences is often, although not always, manifested cytogenetically by the presence of chromosomes containing homogeneously staining regions (HSRs), or paired acentric chromatin bodies termed double minutes (DMs) (3). Several studies demonstrate that these chromosomal abnormalities represent the amplification of cellular oncogenes in some tumors and tumor lines. For example, the *cKi-ras* oncogene is amplified approximately 60-fold in the Y1 adrenocortical tumor line, with overproduction of the corresponding mRNAs and p21 protein product (4). In studies of neuroblastoma and a variant class of small-cell lung carcinoma, amplification of a member of the *myc* gene family (*c-myc, L-myc,* or *N-myc*) is frequently associated with tumor progression (5–10). Other examples of oncogene amplification have been reported (for review, see reference 2), and together these studies suggest that the amplification and consequent overexpression of cellular oncogenes plays an important, although still undefined, role in the initiation or progression of certain tumors. Further analysis of tumor cells and cell lines that exhibit DMs or HSRs may reveal the amplification of other known oncogenes, but in the event that the involved sequences have

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not been previously characterized, isolation and functional characterization of these sequences is necessary in order to understand their contribution to tumorigenesis.

Therefore, as part of our studies to develop a better understanding of the relationship between gene amplification and mechanisms of cellular transformation, we are exploring the origin and function of amplified sequences in a spontaneously transformed derivative of a mouse 3T3 cell line (designated 3T3-DM) with an average of 25–30 DMs per cell. The 3T3-DM cells exhibit many properties of transformed cells in culture: the cells reach a high saturation density in culture, grow well in low serum and soft agar, and form tumors in nude mice (our unpublished observations). An initial approach to understand the association of the DMs with the transformed properties of these cells was to isolate DNA sequences from a recombinant DNA library enriched in these DMs and to demonstrate that the DMs result from gene amplification (11). We have obtained evidence that the sequences amplified in the 3T3-DM cells do not include any of 29 previously identified oncogenes, or growth factor or growth factor receptor genes, including: *abl, B-lym, erb, ets, fes/fps, fgr, fms, fos, H-ras, IGF*-II, *int-1, int-2, Ki-ras, met, mos, myb, myc, N-myc, N-ras, neu, pim, raf, rel, ros, sis, src, yes,* and the receptors for *PDGF* and *IGF*-I (our unpublished results).

Here we report the isolation and characterization of cDNA clones that represent two genes amplified and overexpressed in the 3T3-DM cells. Using a panel of somatic cell hybrids and in situ hybridization protocols, we have determined the mouse chromosome from which these sequences were derived. We have also found that these genes which are amplified in the 3T3-DM cells are present and conserved in other mammalian genomes, including those of human and rat.

MATERIALS AND METHODS

Cell Lines. The Balb/c 3T3-DM cells that we are analyzing apparently became transformed spontaneously in culture: they contain an average of 25–30 DMs per cell (11). This cell line was originally selected for

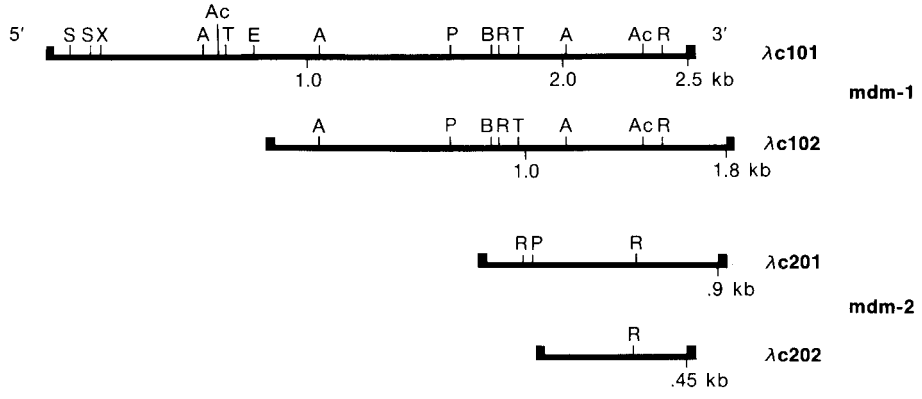

Fig. 1. Partial restriction maps of *mdm-1* and *mdm-2* cDNA clones. Positive cDNA clones were isolated as detailed in Materials and Methods, and insert fragments were subcloned into the EcoRI site of pBR322 for restriction mapping. Assignment of clones to classes and estimation of clone overlap was accomplished on the basis of restriction patterns and cross-hybridization experiments. Not all cDNA clones isolated in the screening procedure are diagramed, but the largest clones are presented above. Each cDNA clone is bounded by EcoRI sites (solid boxes) generated during the cDNA cloning procedure. The *mdm-1* probe p12 comprises the 5′ 0.8 kb EcoRI fragment contained in λc101. The 5′–3′ orientation of *mdm-1* clones was determined from hybridization of strand-specific probes to RNA blots. The *mdm-2* probe pdm66 contains a 1.4 kb PstI-EcoRI genomic fragment isolated from the 3T3-DM DNA library on the basis of homology to λc201 and λc202; both probes are described in more detail in Materials and Methods. The following abbreviations were used for restriction enzymes: A, AvaI; Ac, AccI; B, BamHI; E, EcoRI; P, PstI; R, RsaI; S, StuI; T, TaqI; and X, XhoI.

resistance to bromodeoxyuridine and is thymidine kinase-deficient (12). The Balb/c-derived A31 cell line (provided by Dr. Charles Scher) and the A9 cell line (13) are established and transformed cell lines, respectively, that lack DMs or HSRs. The Y1 mouse adrenocortical tumor cell line has been characterized. Two sublines exist, one of which contains DMs and the other of which contains a large HSR (14). In both sublines the *cKi-ras* gene and at least two other genes are amplified 30- to 60-fold (4, 15). The Rat-2 cell line was obtained from W. Topp (16). The 416B hematopoietic cell line (obtained from E. Scolnick) was used for in situ hybridization experiments because it contains a relatively unrearranged karyotype (17); it has been used successfully for the regional mapping of genes in the mouse (17, 18).

Construction and Screening of 3T3-DM cDNA Library. Polyadenylated (poly-A^+) RNA was prepared from total cellular RNA using either oligo-dT cellulose columns (19) or Hybond mAP (Amersham, Arlington Height, Illinois). The 3T3-DM cDNA library was prepared according to the method of Schwarzbauer et al. (20) and cloned into the vector λgt11. In order to isolate cDNA clones representing genes amplified and overexpressed in this cell line, a two-step screening protocol was utilized. The cDNA library was plated out and transferred in duplicate to nitrocellulose filters and screened in a differential fashion. For one set of hybridizations, radiolabeled first-strand cDNA probe prepared from 3T3-DM or Y1 poly-A^+ RNA was used to screen the library, while for a second set the same probes were prepared but prehybridized with an excess of total A31 RNA before use in hybridization (21). In both cases, plaques were selected for further analysis if a considerably stronger signal was detected with the 3T3-DM probe. These selected phage were then hybridized in duplicate to ^{32}P-labeled genomic DNA from the same cellular sources to determine if their inserts represented DNA sequences amplified in the 3T3-DM cells relative to control mouse cells. DNA inserts in clones scored as positive in these assays were then subcloned into the EcoRI site of pBR322 for further analysis.

Recombinant Probes. Derivation of the probes used in the experiments reported here are indicated in Fig. 1 and described below. Plasmid p12 contains a 5′ 0.8 kilobase pair (kb) cDNA fragment derived from λc101 and inserted in the EcoRI site of pBR322, and represents the *mdm-1* gene (see Fig. 1). The *mdm-1* cDNA fragments hybridize to a number of genomic fragments, some of which had been isolated previously in a random screening of a recombinant library of the 3T3-DM DNA (11). The *mdm-2* gene is represented by the probe pdm66, which contains a 1.4 kb PstI-EcoRI genomic fragment subcloned into pBR322. The cDNAs for the *mdm-2* gene (Fig. 1) contain reiterated DNA that was difficult to separate from single-copy sequence and thus were unsuitable probes for the experiments described below. However, screening of the DM DNA library with *mdm-2* cDNAs led to the isolation of a homologous 6.6 kb genomic fragment that is amplified in the 3T3-DM cells (see Fig. 2). The 1.4 kb PstI-EcoRI fragment in pdm66 is derived from this 6.6 kb genomic DNA fragment.

Hybridization Conditions. Southern and Northern blots were prepared according to described protocols (15, 22). The filters were hybridized in the presence of heparin (23) or, for the somatic cell hybrid blots, in 50% formamide and 7.5% dextran sulfate (24). Probe DNA fragments were labeled with ^{32}P by nick translation (BRL nick-translation kit, Gaithersburg, Maryland) or by oligo-labeling (25). DNA blots were either washed under stringent (0.1× SSC, 65°C) or under moderately stringent (0.5× SSC, 50°C) conditions as indicated (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate). RNA blots were washed in 0.3× SSC at 60°C.

Somatic Cell Hybrids and In Situ Hybridization Analysis. The derivation of the Chinese hamster–mouse somatic cell hybrids used to map the *mdm-1* and *mdm-2* genes has been described (26, 27). In situ hybridizations

were performed as described (28), with a modified staining protocol (29). Both p12 and pdm66 were labeled with [^{3}H]dATP, -dCTP, and -dTTP (Amersham), but the former was nick-translated while the latter was oligo-labeled. The difference in labeling protocol did not affect the final results of the experiments. Both probes were hybridized to metaphase chromosome spreads prepared from the mouse 416B cell line.

RESULTS

Isolation of Sequences Amplified in 3T3-DM Cells. As part of our characterization of the structure and function of the DMs in this transformed 3T3-DM cell line, we constructed a cDNA library in λgt11 from poly-A$^+$ RNA derived from the 3T3-DM cells. The library was screened as described (see Materials and Methods). Of 11,000 recombinant phage screened, 48 were selected for further analysis. Seven of those 48 clones were found to be true positives, representing sequences both amplified and overexpressed in the 3T3-DM cells. The DNA inserts in these cDNA clones were subcloned into pBR322, and by a process of restriction mapping and cross-hybridization experiments were found to fall into two classes, designated *mdm-1* and *mdm-2* (for mouse double minute sequences; see Fig. 1). The *mdm-1* cDNA clones comprise about 2.5 kb of overlapping sequence, while those of *mdm-2* comprise approximately 0.9 kb.

Southern Blotting Analysis. To demonstrate that the cDNA clones we have isolated represent genes amplified in the 3T3-DM cells, Southern blots prepared with EcoRI-digested genomic DNA from these cells and control cell lines were hybridized to *mdm-1* and *mdm-2* probes (Fig. 2). The *mdm-1* probe p12 is the 5′ 0.8 kb EcoRI fragment derived from λc101; the *mdm-2* probe pdm66 is a 1.4 kb genomic fragment isolated on the basis of homology to λc201 and λc202 (for a more detailed description of the probe derivations, see Fig. 1 and "Recombinant Probes" under Materials and Methods). The results obtained are consistent with the conclusion that both probes contain sequences that are amplified in the 3T3-DM cells. For a given probe, all of the mouse cell lines examined exhibit the same pattern of hybridization; however, in the 3T3-DM lanes, the signals are significantly more intense. The degree of amplification of the *mdm-1* and *mdm-2* genes was estimated by a serial-dilution experiment. Comparison of the signals obtained using different amounts of 3T3-DM DNA with that observed for control DNA suggest that both *mdm-1* and *mdm-2*

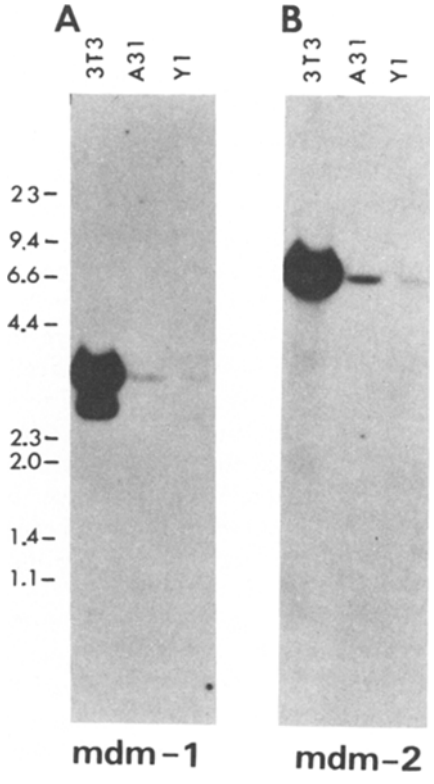

Fig. 2. The *mdm-1* and *mdm-2* sequences are amplified in DM-containing 3T3 cells. Five micrograms each of 3T3-DM, Y1, and A31 genomic DNA were digested with EcoRI and separated on a 1% agarose gel. In panel A, *mdm-1* probe p12 detects genomic fragments of 3.3 and 2.7 kb, while in panel B *mdm-2* probe pdm66 detects a single 6.6 kb fragment. The 3.3 and 2.7 kb fragments in the control lanes of panel A are more clearly visualized on longer exposure of this autoradiogram. Molecular weight markers derived from HindIII-digested λDNA and HaeIII-digested φX 174 DNA are indicated to the left.

are amplified approximately 25-fold in the 3T3-DM cells (not shown).

Expression of Amplified Sequences. Northern blots were used to obtain information on the size and relative abundance of RNA species produced by these amplified genes. As shown in Fig. 3A, *mdm-1* probe p12 detects a major 3.1 kb RNA species and a minor species of 2.4 kb in the 3T3-DM cells; these same RNAs are evident in controls only after a much longer exposure (Fig. 3B), indicating that these sequences are overexpressed at least 25- to 50-fold in the 3T3-DM cells. It is possible that these two transcripts result from differential use of promoters, polyadenylation signals, or alternative splicing events. Hybridization of *mdm-2* probe pdm66 to a similar Northern blot reveals that a 3.4 kb RNA species is detected in the 3T3-DM cells and in control cells (Fig. 3C). This RNA species is also at least 25-fold more abundant in the 3T3-DM cells than in the controls. Both the *mdm-1* and *mdm-2* transcripts are detected in a variety of cell types and in nontransformed cell lines (Fig. 3 and our unpublished observations), suggesting that expression of these sequences may be important for basic cellular functions.

Origin of Amplified Sequences. To establish whether the amplified sequences are associated with the DMs, we utilized a differential centrifugation protocol to prepare a chromosome fraction enriched for DM particles (15). DNA derived from this preparation was digested in parallel with serial dilutions of 3T3-DM total genomic DNA. When *mdm-1* probe p12 is hybridized to this blot, the expected fragments are detected in total and DM DNA (Fig. 4). However, approximately 50 ng from the DM-enriched preparation of DNA yield a signal similar to that produced by 1 μg of total 3T3-DM DNA, as expected if these sequences are associated with the DMs. The same observations are made when this blot is hybridized to *mdm-2* probe pdm66 (not shown). These results were obtained only with sequences amplified in the 3T3-DM cells. As a control, a *vKi-ras* gene

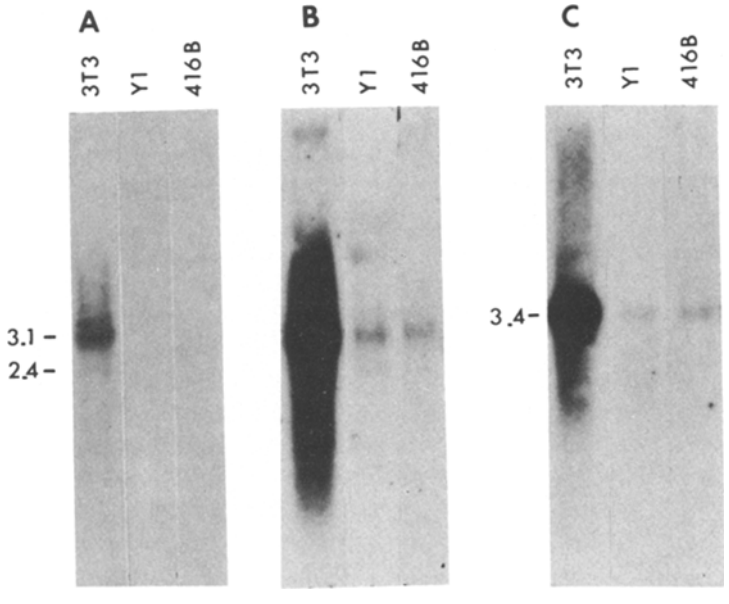

Fig. 3. Transcription of *mdm-1* and *mdm-2* sequences. Two micrograms each of poly-A$^+$ RNA from the 3T3-DM, Y1, and 416B cells were separated on a 1.2% formaldehyde–agarose gel, and the resulting RNA blots were hybridized to *mdm-1* probe p12 (A, B) or *mdm-2* probe pdm66 (C). Panel A is a 5-h exposure, while panel B is a 72-h exposure of the same blot.

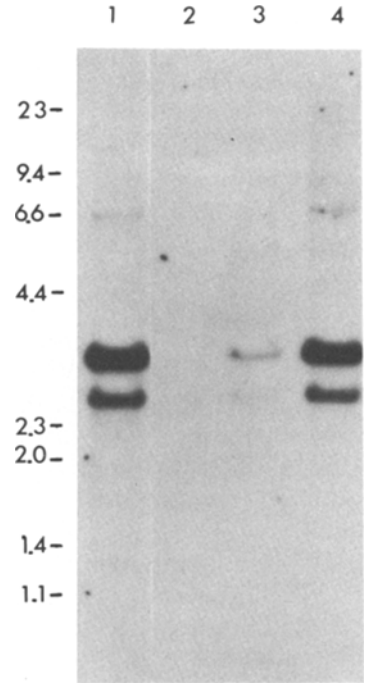

Fig. 4. Enrichment for amplified sequences with DM DNA. Serially diluted 3T3-DM genomic DNA and approximately 50 ng of isolated DM DNA were digested with EcoRI and separated on a 1% agarose gel. The resulting Southern blot was hybridized with p12. Lane 1: isolated DM DNA; lanes 2–4: 10, 100, and 1000 ng, respectively, of 3T3-DM genomic DNA.

probe (pHiHi3, reference 30) was hybridized to the same blot; the *cKi-ras* gene is not amplified in the 3T3-DM cells. Faint hybridization to fragments in the total DNA lane was observed, but no signal was detected with the DM DNA (not shown). These results provide evidence that the genes amplified in the 3T3-DM cells are located on the DMs.

Gene Mapping. Identification of the chromosomal origin of the amplified genes could prove useful in establishing their identity and characterizing their function. To determine from which chromosome(s) the amplified sequences are derived, a panel of Chinese hamster–mouse somatic cell hybrids was screened using the same *mdm-1* and *mdm-2* probes. Figure 5 is a representative Southern blot of the hybrids indicating the mouse and Chinese hamster fragments detected by these probes. Each hybrid was scored for the presence or absence of mouse fragments specific for *mdm-1* and *mdm-2*. Comparison with the mouse chromosome content of each hybrid (Table 1) revealed that, for both probes, the mouse-specific fragments segregated concordantly with mouse chromosome 10. There is one discordancy for chromo-

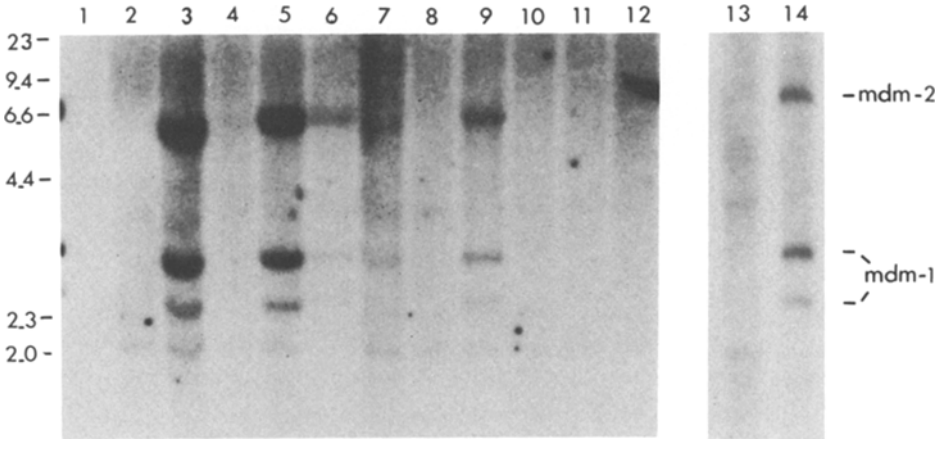

Fig. 5. Detection of genomic fragments homologous to *mdm-1* and *mdm-2* in Chinese hamster–mouse somatic cell hybrids. Each lane contains 10 μg of EcoRI-digested genomic DNA from the following sources. Lanes 1–12: hybrids EAS5-7c, EAS5-2a, EBS11, EBS18, EBS82, I-18a, EBS58, I-13a-1a aza, I-3-2D-1d aza, EAS5-4b, EBS4, and EBS10, respectively. Lanes 13 and 14 contain Chinese hamster and mouse (A9) controls, respectively. The blot was hybridized simultaneously to *mdm-1* and *mdm-2* probes. Indicated to the right of the blot are the mouse genomic fragments detected by the probes.

Table 1. Hybridization of DM Probes with Chinese Hamster × Mouse Hybrid DNA and Mouse Chromosomes Retained: Mapping to Mouse Chromosome 10

Hybrid	mdm-1 and mdm-2[a]	Mouse chromosomes[b]																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X
I-3-2D-1d aza	+	+	+	−	+	−	+	+	+	+	+	−	+	+	+	+	P	+	+	+	P
I-10C HAT	+	+	+	+	+	−	+	+	+	L	+	L	+	−	+	+	+	+	+	+	+
I-13A-1a aza	−	+	+	+	+	−	+	+	+	+	−	−	+	+	+	+	+	+	+	+	P
I-13A-2a aza	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	−	+	+	+	−
I-18A HAT	+	+	+	−	+	−	+	−	−	−	+	−	+	−	−	−	+	+	L	+	+
I-18A-2a aza	−	+	+	−	+	+	+	−	−	−	−	−	+	−	−	−	−	L	+	+	−
EAS5-2a	−	+	+	+	−	+	+	P	+	−	−	−	−	−	+	+	+	+	−	+	+
EAS5-4b	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+	+	+	+
EAS5-7c	−	−	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	+	−	+	+
EBS4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	+
EBS10	−	+	+	+	−	−	L	+	−	L	+	−	+	L	+	+	+	−	+	−	+
EBS11	+	+	−	−	−	−	−	+	−	−	+	−	+	−	−	+	+	−	−	−	+
EBS18	−	−	+	+	−	−	+	+	L	−	L	−	+	−	−	+	−	+	−	−	−
EBS51	−	L	+	+	L	L	+	L	+	+	−	−	+	+	−	+	L	+	+	+	+
EBS58	+	+	+	+	+	−	L	+	−	−	+	−	+	+	−	+	−	+	−	+	+
EBS82	+	L	+	L	−	−	−	+	−	+	L	−	+	−	L	+	L	+	−	+	+
EZS25	−	−	+	−	+	−	−	+	+	−	−	−	+	+	−	+	+	+	L	+	+
RTM9	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	−
Ratio of discordancies[c]		6/16	11/18	11/17	8/17	11/17	10/17	8/16	11/17	6/16	1/16	6/17	7/18	9/17	8/17	11/18	6/15	10/17	9/16	9/18	7/16

[a]Presence of genes determined by scoring the presence (+) or absence (−) of the respective mouse DNA fragments.
[b]L = chromosome present at low frequency; P = only part of chromosome present.
[c]Number of discordant hybrids/total number of informative hybrids excluding L and P data; discordancy was scored as presence of mouse-specific fragments but not a specific chromosome, or the reverse situation.

some 10 (hybrid EBS10), in which no hybridization signal is detected but an apparently normal chromosome 10 is present in the hybrid cells. It is possible that this chromosome 10 contains a small rearrangement not detectable by chromosome analysis that might account for these results. For all other chromosomes except 10, there were six or more discordant clones.

To confirm that *mdm-1* and *mdm-2* sequences are derived from mouse chromosome 10, and to define the subchromosomal location of these genes, in situ hybridization experiments were performed using metaphase chromosomes prepared from the 416B mouse cell line. The 416B cells were especially useful because they contain a Robertsonian translocation involving chromosomes 2 and 10. For each probe analyzed, approximately equal numbers of grains were found to be present on the translocated chromosome 10 as the normal 10. Figure 6 presents results from these experiments. For *mdm-2* probe pdm66, we examined 127 metaphases containing 183 grains over chromosomes. Of 183 grains, 36 (19.7%) hybridized to chromosome 10, and 60% of these were assigned to region C1-C3. Similar results were obtained for *mdm-1* probe p12. Of 59 grains on 51 metaphases examined, 24% of the grains hybridized to chromosome 10, and 79% of those grains could be assigned to region C1-C3. For both probes, the grains not located on chromosome 10 were randomly distributed over the other chromosomes (data not shown).

As noted above, in previous experiments we had obtained evidence that none of 29 oncogenes examined were amplified in the 3T3-DM cells. The mapping data presented here provide additional support for this conclusion. Although the *myb* protooncogene has been assigned to mouse chromosome 10 (31), we have found that it is not amplified in the 3T3-DM cells (data not shown).

Evolutionary Conservation of mdm-1 and mdm-2. Genes that are important for cellular growth, growth control, and development are frequently conserved evolutionarily.

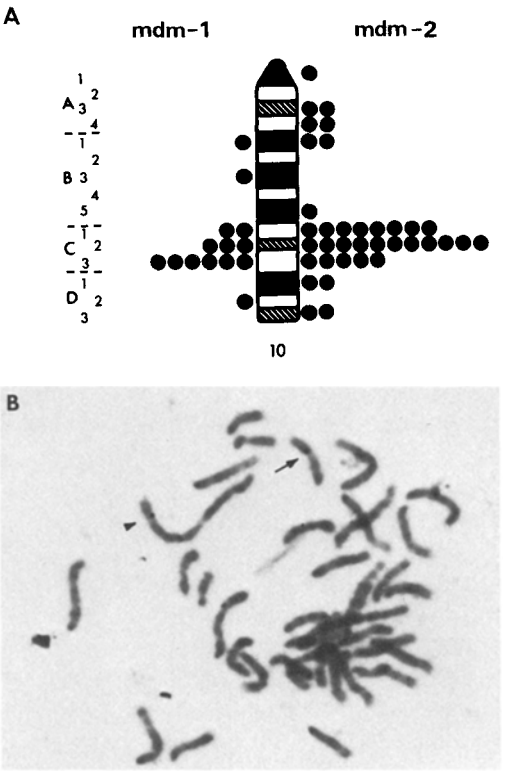

Fig. 6. Regional localization of *mdm-1* and *mdm-2* genes by in situ hybridization. (A) An idiogram of mouse chromosome 10 is depicted (32), with solid circles indicating the distribution of labeled sites for *mdm-1* and *mdm-2*. A representative metaphase spread is presented in B. The arrow and arrowhead indicate hybridization of pdm66 to the normal and translocated chromosomes 10, respectively, in the 416B cells.

To determine whether the *mdm-1* and *mdm-2* genes might be so conserved, we hybridized p12 and pdm66 to mouse, rat, and human genomic DNA. Figure 7 presents data for p12. Under moderately stringent filter wash conditions, cross-hybridizing fragments are clearly detected in both the rat and human lanes. Similar results were obtained for the *mdm-2* probe pdm66 (not shown). These data indicate that the two DM-associated genes represent evolutionarily conserved sequences.

DISCUSSION

We have utilized a differential screening protocol to isolate cDNA clones representing

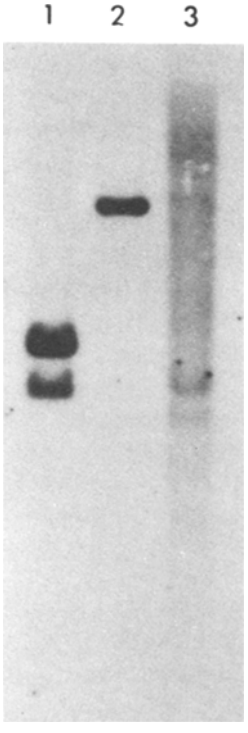

Fig. 7. Conservation of amplified sequences in rat and human DNA. In lanes 1–3, 10 μg each of mouse A9, Rat-2, and human DNA, respectively, were digested with EcoRI and separated on 1% agarose. The resulting blot was probed with p12 and washed under moderately stringent conditions at 50°C in 0.5× SSC.

two genes amplified in a spontaneously transformed mouse 3T3-DM cell line. Both genes are amplified approximately 25-fold and overexpressed in the 3T3-DM cells relative to control cells. Hybridization experiments reveal that the amplified genes are associated with the DMs and were derived from mouse chromosome 10, region C1-C3. Although no known oncogenes are amplified in the 3T3-DM cells, the sequences we have isolated are evolutionarily conserved among mouse, rat, Chinese hamster, and human genomes, and they are expressed in cell lines having myeloid, adrenocortical, and fibroblastic origins, indicating that they may represent sequences involved in one or more basic cellular functions. We have compared initial DNA sequence data obtained for *mdm-1* with nucleic acid sequence data bases and, to date, no homologies have been detected (our preliminary data). Future studies may include appropriate transfection experiments to assess the function of the genes amplified in the 3T3-DM cells, in addition to more complete characterization of the DNA sequence and protein products of the *mdm-1* and *mdm-2* genes.

Based on their physical size, DMs could contain approximately 1000–2000 kb of DNA (2, 3). At present we have no information as to the locations of *mdm-1* and *mdm-2* on the DMs relative to each other or to other genes that most likely are present on these structures. Such information could be obtained by analyzing larger segments of DNA, using techniques such as pulsed-field gradient-gel electrophoresis.

We are not aware of data that would implicate mouse chromosome 10, region C1-C3, in the etiology of neoplasms in the mouse. The contribution that one or more of the amplified genes may make to the transformed properties of the 3T3-DM cells remains to be determined. However, given that the DMs are stably maintained in the 3T3-DM cells in the absence of any obvious selection pressure, and that amplified DNA sequences in a number of other tumor cell types have been shown to include previously identified cellular oncogenes, it is possible that continued study of the amplified sequences in the 3T3-DM cells may lead to the isolation of a previously unidentified cellular gene with potential transforming activity.

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