A Gene Amplified in a Transformed Mouse Cell Line Undergoes Complex Transcriptional Processing and Encodes a Nuclear Protein*

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We have explored the structure and pattern of expression of a gene designated mdm-1, which is amplified 25–30-fold in transformed mouse cells containing numerous double minute particles. This gene is expressed in all mouse tissues examined but exhibits elevated and altered patterns of expression in the testis. Multiple transcripts are generated from the mdm-1 gene via mechanisms of alternative splicing and polyadenylation signal choice. These mRNAs have the potential to produce a minimum of three distinct protein products ranging in size from 25 to 77 kilodaltons. Antiserum generated against a synthetic peptide from the mdm-1 gene was used in immunoblotting studies and revealed that at least one of the protein products is present in the nucleus. This antiserum stained nuclear structures producing a distinct punctate or speckled pattern.

A number of genes have been implicated in altered growth control pathways associated with cellular transformation (Bishop, 1987). Clues leading to the identity of some of these genes have come from cancer cells themselves in the form of consistent chromosomal changes. These changes include specific deletions and translocations, as well as the appearance of double minute chromatin particles (DMs) in some tumor cells. DMs are small,acentromeric chromatin bodies that result from a process of gene amplification. DMs are rarely, if ever, observed in normal cells but have been described in a variety of tumor cells and transformed cell lines (George, 1984), and in cells selected for resistance to various antime-tabolites (Schimke, 1984; Stark and Wahl, 1984). A demonstration that the appearance of DMs results from the aberrant replication and/or recombination of a segment of genomic DNA initially came from studies of mammalian cells selected for resistance to the drug methotrexate (Alt et al., 1978). Analysis of such methotrexate-resistant cells, as well as cells selected for resistance to a number of other drugs, has provided evidence that cells containing DMs most likely are selected for and maintained in the population because the DMs contain amplified copies of one or more genes, which confer resistance to the cytotoxic agent. Removal of the drug, and the selective pressure it imparts, results in rapid loss of DMs (Alt et al., 1978; Kaufman et al., 1978).

In contrast, DMs are stably maintained in tumor cells in the absence of obvious selective pressure. Therefore, it is likely that the amplified DNA on the DMs confers some growth advantage to the host cell. Indeed, amplified sequences present in a number of tumor cells and transformed cell lines often include previously characterized proto-oncogenes or related genes (George, 1984). These data suggest that the amplification and consequent overexpression of particular genes may play a central role in some processes of transformation.

We are exploring the functional significance of amplified DNA sequences located on DMs in a spontaneously transformed mouse cell line. These 3T3DM cells contain an average of 25–30 DMs and exhibit growth properties characteristic of transformed cells, including growth in soft agar and tumor formation in nude mice (Cahilly-Snyder et al., 1987). We have obtained evidence that none of 30 previously identified oncogenes, growth factors, or growth factor receptor genes are amplified in these cells (Cahilly-Snyder et al., 1987). However, the DMs are stable in the 3T3DM cells and therefore may well contain a previously unidentified gene with potential transforming activity or influence in the control of cellular proliferation. To begin to characterize the origins and nature of the amplified DNA sequences, we had previously constructed a 3T3DM cDNA library and isolated clones representing two genes that are amplified and overexpressed in these cells (Cahilly-Snyder et al., 1987). These two genes, designated mdm-1 and mdm-2, are amplified approximately 25-fold relative to control cells. As reported elsewhere, in normal cells these genes map to mouse chromosome 10, region C1–C3 (Cahilly-Snyder et al., 1987). This region of the genome has not been previously implicated in transformation or altered growth control.

As one approach to further characterize the amplified material in the 3T3DM cells, we have analyzed the sequence and pattern of expression of the amplified mdm-1 gene. As presented in this report, we have found that the mdm-1 gene is subject to complex transcriptional processing, with the potential to produce a number of polypeptide products. At least one of these proteins is present in the nucleus.

MATERIALS AND METHODS

Cells and Cell Lines—The 3T3DM cells are a spontaneously transformed, BALB/c-derived cell line (Matsuya and Green, 1969). The Y1 mouse cell line was derived from an adrenocortical tumor and...
exhibits amplification of DNA sequences, including the cKi-ras oncogene (Schwab et al., 1983); sequences amplified in the 3T3DM cells are different from those amplified in the Y1 cells (George and Powers, 1981; Schwab et al., 1983). The BALB/c-derived A31 cell line (a gift of Dr. Charles Scher, University of Pennsylvania) is an established but nontumorigenic line that does not exhibit gene amplification.

**Nested Probes and cDNA Clones**—The mdm-1 cDNA clones Xc101 and Xc102 were isolated from the 3T3DM cDNA library as described previously (Cahilly-Snyder et al., 1987). The 5'-most 0.7-kb EcoRI fragment of Xc101 (see Fig. 1), designated p12, was used to rescreen this library and led to the isolation of clones Xc103 and Xc105. Clone Xc104 was isolated from a second 3T3DM cDNA library also on the basis of homology to p12; this library was constructed essentially as described by Guibler and Hoffman (1983) in ZAP (Stratagene).

**DNA Sequence Analysis**—DNA fragments were sequenced by the method of Sanger et al. (1977). Portions of cDNA or genomic fragments were subcloned into either m1508p18 and m19 or into the polylinker of pGEM4 (Promega) for sequencing using the SP6 and T7 promoter-specific primers (Promega). The 5'-most 0.7-kb EcoRI fragment of Xc101 was used to amplify cDNA sequences, including the cKi-ras oncogene (Promega), and then dried down for autoradiography. These sequences were separated on 10% SDS-polyacrylamide gels (Laemmli, 1970), blotted onto nitrocellulose (Towbin et al., 1979) for probing with preimmune and immune sera incubated with products of the in vitro transcription and translation, and immune complexes were separated using washed Staphylococcus aureus (Enzyme Center, Kessler, 1975). For the Western blots, cells were fractionated as described (Ikegaki et al., 1986) and the protein quantitated using the Bio-Rad protein assay kit. Nuclear and cytoplasmic components were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose (Towbin et al., 1979) for probing with preimmune and immune serum. Immune or preimmune serum was diluted 100-fold before incubation with the Western blots. To visualize immune complexes, the Vectastain ABC kit was employed.

**Indirect Immunofluorescence**—Immunofluorescence studies were performed as described previously (Dlugosz et al., 1984). Briefly, 3T3DM cells were grown on Corning 35-mm tissue culture dishes, rinsed in phosphate-buffered saline, and fixed in 20°C methanol for 5 min and air-dried. The wash buffer used below contained 0.5% Triton X-100 and 5% normal goat serum in phosphate-buffered saline and was also used for blocking and dilution of antisera. Cells were incubated with wash buffer and subsequently incubated with immune or preimmune serum diluted 1:300. After three 20-min washes, the cells were incubated with affinity-purified rhodamine-conjugated goat anti-rabbit IgG (Cappel) diluted 1:100 prior to use.

**RESULTS**

**Sequence of mdm-1 cDNA Clones**—As part of our characterization of the DMs in the 3T3DM cells, we have analyzed the structure and expression of one of the amplified genes, mdm-1. Use of mdm-1 probes in Northern blot analysis revealed hybridization to poly(A)+ RNA of approximately 3.1, 2.4, and 1.4 kb in size (Cahilly-Snyder et al., 1987; see also Fig. 6, lane 5). Transcripts of the same size are seen in other cells and normal tissues but are present at 50-500-fold higher levels in the 3T3DM cells. Several lines of evidence demonstrate that the various transcripts are derived from a single mdm-1 locus. All of the mdm-1 cDNAs and genomic DNA probes we have isolated hybridize to contiguous genomic fragments contained within a single cosmID clone (data not shown). Moreover, Southern blot hybridization of the cDNAs and genomic fragments of the mdm-1 locus led to the discovery of a single cosmID clone that contains the mdm-1 gene.
FIG. 2. Nucleotide sequence derived from mdm-1 cDNA clones Xc101, Xc102, and Xc103. The putative protein sequence is translated underneath the nucleotide sequence. Nucleotide and amino acid residues are numbered to the right of each line. The box encloses the 135-base exon that is spliced out in some mdm-1 mRNAs. After base 666, some mdm-1 mRNAs have different 3' ends, denoted by an arrow pointing to the sequence underneath bases 667-720 (derived from cDNA clones Xc104 and Xc105; the three dots within this sequence represent 511 bases not shown here). Stop codons are marked by asterisks. Polyadenylation signals are overlined. The peptide used to generate antisera is identical to residues 409-424 (underlined).

We have sequenced a genomic fragment that contains this 135-base exon and find that the splice junctions (5'-ttttcccccaag/TTG...cag/tgac-3') conform to the consensus sequences for these signals (Mount, 1982).

Additional evidence for the presence of distinct mdm-1 transcripts was obtained from nuclease protection assays. For these studies, we end-labeled cDNA fragments derived from clone Xc101 which spanned the 135 bases missing in clone Xc103. In one experiment, a single-stranded XhoI-Accl fragment (see Fig. 3A) was labeled at the 5' end and hybridized to RNA from the 3T3DM cells and from two control cell lines. The sequencing data in Fig. 2 predicts that two fragments of 463 and 197 bases in size should be protected from digestion. These fragments would represent cDNA clones Xc101 and Xc103, respectively. As shown in Fig. 3B, two bands of the predicted sizes are generated by nuclease digestion. In addition, a third protected fragment of 164 bases is seen (Fig. 3B), indicating that mdm-1 may be producing yet another distinct transcript. A cDNA clone corresponding to this putative third transcript has not yet been isolated. When these same experiments were performed using RNA isolated from two control cell lines, which do not exhibit amplification or overexpression of mdm-1, similar results were obtained (Fig.

and genomic clones to mouse genomic DNA under low stringency conditions does not reveal additional DNA fragments that might be associated with related genes (data not shown). Results from gene mapping studies also provided evidence that there is only a single mdm-1 locus in normal mouse cells (Cahilly-Snyder et al., 1987).

In order to define the relationship among these transcripts, as well as to identify and characterize potential open reading frames, we sequenced a number of mdm-1 cDNA clones isolated by screening two independent 3T3DM cDNA libraries (see Fig. 1). Within the sequence derived from overlapping clones λc101, λc102, and λc103, a long open reading frame could be identified. It extends from an ATG codon at base 22 to a TAA stop codon at base 2088 (Fig. 2). The sequence predicts that mdm-1 could encode a protein of 689 amino acids. In addition, the length of the sequence (2925 bases) suggests that it is derived from a 3.1-kb mdm-1 mRNA species.
Structure and Complex Expression of an Amplified Gene

Fig. 3. Nuclease protection assays to map alternate splicing events in m<sub>dm</sub>-1 mRNAs. A, derivation of probes used in nuclease protection assays. The probes are derived from clone λc101. The filled box denotes the 135-base (b) exon. Open boxes represent plasmid sequences that permit distinction between the probe fragment and the largest protected fragment in the assay. Sizes of probes and protected fragments are indicated. Asterisks represent location of label on the fragment. X, XhoI; A, AccI; E, EcoRI. B, protected fragments generated by hybridization of 540-base XhoI-AccI 5'-end-labeled probe to 3T3DM and control RNAs. Lane 1, no added RNA; lanes 2–5, 20 μg of tRNA, 3T3DM RNA, Y1 RNA, or A31 RNA, respectively. Lanes 1–3 represent a 3-day exposure, while lanes 4–5 represent a 40-day exposure of the autoradiogram. Molecular weight markers are denoted by dashes to the left of panels B and C and are 32P-end-labeled fragments generated by HpaII digestion of pH922 DNA (622, 527, 404, 309, 242, 258, 217, 201, 190, 180, 160, 147, and 122 bases in size). C, protected fragments generated by hybridization of 570-base XhoI-EcoRI 3'-end-labeled fragment to 3T3DM RNA. Lane 1, no added RNA; lane 2, 20 μg of tRNA; lane 3, 20 μg of total RNA from the 3T3DM cells.

3B, lanes 4–5). Therefore, the presence of multiple m<sub>dm</sub>-1 transcripts is not an abnormal consequence of the amplification event but rather represents a normal pattern of expression for this gene.

Further information on potential boundaries of spliced exons was obtained when the nuclease protection assay was repeated using a single-stranded XhoI-EcoRI fragment labeled at the 3' end instead (see Fig. 3A). Again, three protected fragments are detected in the 3T3DM lane (Fig. 3C). The 540-base fragment represents the λc101-like message, while the 129-base fragment is of the size expected for the λc103-like message. An additional protected fragment is evident at 265 bases. This band is derived from the 1.4- and 2.4-kb m<sub>dm</sub>-1 mRNAs, as discussed below.

Sequencing of cDNA clones λc104 and λc105 revealed that m<sub>dm</sub>-1 may encode yet another distinct polypeptide and provided information on the relationship among the various m<sub>dm</sub>-1 mRNA species. Northern blot analysis using a 5' m<sub>dm</sub>-1 probe reveals hybridization to RNAs of approximately 3.1, 2.4, and 1.4 kb in size (Cahilly-Snyder et al., 1987; see also Fig. 6). The sequence of cDNA clones λc101, λc102, and λc103 shown in Fig. 2 most likely is derived from the "3.1-kb" m<sub>dm</sub>-1 transcripts. As described below, the λc104 and λc105 cDNA clones represent the 2.4- and 1.4-kb mRNAs. The sequence of clone λc105 is entirely contained within λc104 (Fig. 1), so further discussion will be restricted to clone λc104. We found that the nucleotide sequence of λc104 is identical to that of clone λc101 up to base 666, after which point the sequences diverge (see arrow, Fig. 2). When the genomic DNA in this region was analyzed, we found that the 3' terminus of λc104 is actually contained within what would be an intron for the 3.1-kb mRNAs (see model in Fig. 4). In the cDNA and genomic clones, an in-frame termination codon (TGA) is present seven codons downstream of base 666, and a polyadenylation signal (AATAAA) is present 514 bases 3' of the stop codon. In the λc104 cDNA, a poly(A) tail is present 18 bases 3' of the AATAAA (see Fig. 2). Thus, m<sub>dm</sub>-1 has the potential to produce another smaller protein of 222 amino acids, with a carboxyl terminus distinct from that encoded by the 3.1-kb mRNAs.

With reference to the 2.4-kb mRNA, a full length cDNA clone has not yet been isolated. However, Northern blotting experiments suggest that the 2.4-kb transcript is structurally related to the 1.4-kb message. All m<sub>dm</sub>-1 transcripts, 3.1, 2.4, and 1.4 kb, are detected by a 5' cDNA probe as shown in Fig. 6. However, if probes 3' of the EcoRI site in clone λc101 (Fig. 1) are hybridized to 3T3DM RNA, only the 3.1-kb messages are detected. In contrast, if a probe derived from the 3'-nontranslated region of clone λc104 (Real-EcoRI fragment in Fig. 1) is used in Northern blotting studies, both the 2.4- and 1.4-kb mRNAs, but not the 3.1-kb mRNAs, are detected (data not shown). The results are consistent with the possibility that the 2.4- and 1.4-kb mRNAs share at least some coding material but differs in the lengths of the 3'-nontranslated region. However, isolation of additional cDNA clones is needed to confirm this model.

These studies demonstrate that m<sub>dm</sub>-1 can generate multiple mRNAs through the mechanisms of alternative splicing and/or polyadenylation signal choice. The m<sub>dm</sub>-1 transcripts have the potential to encode a number of polypeptide products. The 3.1-kb mRNAs detected by m<sub>dm</sub>-1 probes in Northern blots include the alternatively spliced λc101-like and λc103-like transcripts, which should encode a protein of 689 amino acids and a smaller polypeptide, respectively. In contrast, translation of the 1.4-kb (and perhaps 2.4-kb) tran-

Fig. 4. Schematic diagram depicting our current working model for generation of various m<sub>dm</sub>-1 mRNAs through alternative splicing and/or polyadenylation signal choice. The top line represents m<sub>dm</sub>-1 genomic DNA; open boxes are exons, and bold lines are introns. Below are the different m<sub>dm</sub>-1 mRNAs. The 3.1-kb mRNAs may include or exclude the exon indicated. The 1.4-kb mRNA utilizes a polyadenylation signal present in an intron removed from the 3.1-kb mRNAs. Preliminary data indicate that the 2.4-kb mRNA differs from the 1.4-kb mRNA in the length of the 3'-nontranslated region, although further work is required to confirm this. The hatched region represents alternate protein coding material (seven amino acids) used by the 2.4- and 1.4-kb mRNAs. aa, amino acids.
scripts should yield a related, yet distinct, 222-amino acid protein.

The 5' End of mdm-1—To define the 5' boundary of the mdm-1 transcripts, and to determine if the ATG at base 22 of the sequence shown in Fig. 2 represents the translational start codon, primer extensions and nuclease protection assays were employed. For primer extension experiments, we used a synthetic oligonucleotide complimentary to nucleotides 41–61 of the sequence shown in Fig. 2. When hybridized to 3T3DM RNA, several major extension products are synthesized, migrating at 147–153, 167, and 172 bases (see Fig. 5B). Thus, the mdm-1 transcripts extend 110–135 bases upstream of the ATG at base 22. The same primer extension pattern is generated by RNAs from control cell lines (Fig. 5B, lanes 2 and 3). The much longer exposure time required to detect the extension products in the control lanes reflects the 30–50-fold lower abundance of the mdm-1 transcripts in these cells compared to the 3T3DM cells.

To confirm these results and to map the relative position of the 5' end of the gene, the nuclease protection assays were utilized. A 618-bp genomic DNA fragment was isolated on the basis of homology to the 5' end of cDNA clone λc103 and sequenced (see Fig. 5A). From this genomic clone, a 190-base StuI-BbU fragment containing the 5'-most ATG of cDNA λc103 and 5' flanking sequence was end-labeled and hybridized to RNA from the 3T3DM cells. As shown in Fig. 5C, a series of protected bands in the size range 110–130 bases is detected. The sizes of the protected fragments are consistent with the data obtained in the primer extension analysis (open and closed circles, Fig. 5A), and indicate that transcription initiates at multiple sites within a region located approximately 110–135 bases upstream of the ATG codon. No other ATG is encountered upstream of this codon; thus, it remains the most likely site for translational initiation. Finally, since transcription appears to initiate within this region, the 618-bp genomic fragment most likely contains at least part of the mdm-1 promoter. No CCAAT or TATA boxes are evident within 400 bases upstream of the transcriptional start region, but this genomic region has a high G+C content (66%) and in fact contains several "GC boxes" (TGGGCGGGGC or its complement, Kadonaga et al., 1986; see Fig. 5A, underlines). These GC boxes represent potential binding sites for the

![Fig. 5. The 5' end of mdm-1. A, sequence of a 618-bp RsaI genomic fragment containing the 5' end of the mdm-1 gene. The first ATG is marked by a thick underline. Transcriptional initiation sites mapped by primer extension are indicated by open circles; those mapped by nuclease protection are denoted by closed circles. Four potential Sph binding sites are indicated by thin underlines. BbU and StuI sites used to generate the probe for nuclease protection assays are marked. The arrow underneath the sequence indicates the splice junction between the first exon and the first intron. B, mapping of the 5' end of mdm-1 by primer extension. A primer complimentary to bases 41–61 in Fig. 2 was hybridized to various RNA samples and extended using reverse transcriptase. Each lane contains 20 μg of RNA derived from 3T3DM, Y1, or A31 cells (lanes 1–3, respectively). Lane 1 represents a 1-day exposure, while lanes 2–3 represent a 12-day exposure. Sizes (in bases) of major primer extension products are indicated to the right of the panel. The marker lane (M) contains end-labeled fragments obtained from HpaII-digested pBR322 (see the legend to Fig. 3B for sizes). C, mapping of the 5' end of mdm-1 by nuclease protection. A 190-base StuI-BbU fragment was end-labeled and hybridized to the following RNA samples: lane 1, no RNA; lane 2, 5 μg of 3T3DM poly(A)+ RNA; lane 3, 20 μg of Y1 poly(A) RNA. Marker fragments are those used in B.]
transcription factor Sp1 (Dyman and Tjian, 1985; Kadonaga et al., 1988); their functional significance with respect to expression of the mdm-1 gene remains to be determined.

Expression of mdm-1 in Mouse Tissues—Previous Northern blotting experiments had indicated that mdm-1 is expressed in a range of mouse cell lines, including those of myeloid, adrenocortical, and fibroblastic origin (Cahilly-Snyder et al., 1987). We extended these studies to investigate the pattern of expression of mdm-1 in different tissues. RNA was extracted from various tissues of adult BALB/c mice and hybridized to mdm-1 5' probe p12 in Northern blots. The p12 probe is derived from λ1101 (see Fig. 1 and "Materials and Methods") and detects all mdm-1 mRNAs. Results from these experiments demonstrate that mdm-1 is expressed at low levels (<0.01% of the mRNA) in all cells and tissues examined (Fig. 6). A level of expression similar to that seen with placenta and thymus (Fig. 6, lanes 2 and 3) was observed using RNAs from heart, brain, lung, liver, kidney, and spleen (data not shown). One difference we did observe involved analysis of adult mouse testis (Fig. 6, lane 4). In this tissue, the mdm-1 mRNAs are at least 5-fold more abundant. Moreover, the 2.4- and 1.4-kb messages are present at a higher level relative to the 3.1-kb mRNAs than we have observed for the 3T3DM cells (Fig. 6, lane 5), as well as all other cells and tissues examined thus far. This raises the possibility that mdm-1 may be differentially expressed during spermatogenesis.

Protein Products of mdm-1—On the basis of the putative amino acid sequence of mdm-1, a hydropathicity plot was generated (data not shown). This plot indicates that mdm-1 has the potential to produce quite hydrophilic proteins, most likely due to the presence of a greater than average number of basic residues in the polypeptides (Doolittle, 1987). Further examination of the amino acid sequence does not reveal other notable features, such as obvious transmembrane domains or signal peptides. The nucleotide and amino acid sequences of mdm-1 do not show significant homology with previously reported sequences stored in the GenBank database.

*In vitro* transcription and translation studies were carried out to begin characterizing the protein products of mdm-1. Coding material sufficient to produce the largest mdm-1 protein was cloned into pGEM4, under the transcriptional control of the SP6 promoter (see Fig. 7A and "Materials and Methods"). *In vitro* transcription and translation reactions gave the results shown in Fig. 7B. The major protein produced in this assay has an approximate molecular mass of 82 kDa (lane 2), which correlates well with the mass of 77 kDa

![Fig. 7. Characterization of in vitro translated mdm-1 mRNA.](image-url)

**Fig. 7.** Characterization of *in vitro* translated mdm-1 mRNA. A, the mdm-1 construct used to generate *in vitro* transcripts. The solid line represents the longest open reading frame of mdm-1 derived from the sequence shown in Fig. 2. Initiation and stop codons are indicated. E/F refers to a Fnu4HI site 13 bases upstream of the ATG which was blunted and ligated to EcoRI linkers for cloning into the EcoRI site. X is an XbaI site present 578 bases downstream of the stop codon. Sense (SP6) or antisense (T7) transcripts were synthesized. N and H represent NaeI and HindIII sites that were cleaved in order to generate discrete transcripts. B, translation of mdm-1 *in vitro* transcripts and immunoprecipitation. SP6 (sense) and T7 (antisense) transcripts synthesized from the construct in A were translated in rabbit reticulocyte lysate in the presence of [35S]methionine and then immunoprecipitated. Lane 1, no mRNA added; lanes 2–4, translation of SP6 transcripts; lanes 5–6, translation of T7 transcripts. Samples in lanes 3 and 6 were immunoprecipitated with antiserum generated against an mdm-1-specific peptide, while the sample in lane 4 was immunoprecipitated with preimmune serum.
predicted for the 689 amino acid polypeptide. This 82-kDa protein is immunoprecipitated by antiserum generated against a peptide identical to residues 409–424 of the predicted mdm-1 amino acid sequence, but not by preimmune serum from the same rabbit (see Fig. 7B, lanes 3 and 4). In contrast, translation of antisense transcripts generates polypeptides that are neither of the predicted size for the mdm-1 protein product nor precipitated by the immune serum.

This antisera was used in Western blotting experiments to gain information on the subcellular location of the mdm-1 protein(s) and to determine the size of the products detected (Fig. 8). Nuclear and cytoplasmic extracts were prepared from 3T3DM and control cells, and equivalent amounts of protein were separated on Laemmli gels. On the resulting Western blots, the immune serum detects a protein of approximately 82 kDa present in the nuclear extracts of the 3T3DM and control cells (see lanes 7 and 8). This protein is not detected with the preimmune serum (Fig. 8, lanes 1–4). From the relative intensity of the bands in lanes 7 and 8, the 82-kDa protein appears to be more abundant in the 3T3DM cells, which would be consistent with the fact that the mdm-1 gene is amplified and overexpressed at the RNA level. Considering the results from in vitro transcription and translation (Fig. 7), we believe that this protein represents the 689 amino acid mdm-1 protein. The peptide was used to generate the antisera before the extent to which mdm-1 undergoes alternative RNA processing was realized; thus, not all mdm-1 proteins may be detected. However, a protein of approximately 77 kDa is detected in cytoplasmic fractions of the 3T3DM and control cells, with a stronger signal in the 3T3DM lane (Fig. 8, lanes 5 and 6). This 77-kDa protein would be consistent with the size expected for a product of the alternatively spliced λCI03-like mRNA. In addition, cytoplasmic proteins of 46 and 21 kDa are detected only by the immune serum. We are currently investigating the relationship of these smaller proteins to the larger 77- and 82-kDa products.

To extend the Western blotting experiments, and to determine the cellular staining pattern produced using the same rabbit anti-mdm-1 serum, we carried out indirect immunofluorescence (Fig. 9). Immunofluorescent analysis of 3T3DM cells with the antisera revealed a distinct speckled or punctate pattern within the nuclei, which is not associated with the nucleoli (Fig. 9B). There is also a less intense, diffuse cytoplasmic staining. This cellular staining pattern was not detected when the assays were carried out using preimmune serum from the same rabbit (Fig. 9D). Treatment of control mouse (Y1) cells with the same anti-mdm-1 sera revealed a similar, although fainter, staining pattern (data not shown).

**DISCUSSION**

We have analyzed the structure and pattern of expression of a gene (mdm-1) amplified and overexpressed in a transformed mouse cell line, in which the amplified copies are located on the DMs. Our data demonstrate that mdm-1 generates several mRNAs that result from alternative splicing of exons and/or differential use of polyadenylation signals. These transcripts have the potential to encode at least three distinct protein products.

The various transcripts encoded by this gene are expressed in a wide range of tissues as well as in a number of cell lines examined to date, at levels 30–50-fold less than those seen in the 3T3DM cells in which the gene is amplified. The one difference we have found in the pattern of expression of this gene involves the testis (Fig. 6). Since germ cells comprise about 90% of the adult mouse testis (Bellve et al., 1977), it is possible that differential expression of the mdm-1 gene may be associated with various stages of germ cell differentiation. Additional studies are in progress to determine if the transcripts are of germ cell origin and if there are qualitative and/or quantitative changes in mdm-1 expression during spermatogenesis. There is some evidence that the testis may possess tissue-specific factors that influence transcriptional processes. For example, in this tissue a unique c-abl mRNA is synthesized through the use of a cryptic polyadenylation signal, although the reading frame of the protein is not disturbed by this event (Meijer et al., 1987). The functional significance of this c-abl mRNA also exhibits increased stability compared to the larger c-abl transcripts that are normally generated by this gene (Meijer et al., 1987). In addition, the chicken β-tubulin gene is expressed in a broad range of tissues, but the transcripts are more abundant in the testis (Havercroft and Cleveland, 1984). Such testis-specific factors may also influence the expression of the mdm-1 gene in this tissue.

Alternative splicing and polyadenylation signal choice are well documented mechanisms of transcriptional processing control, which permit a single gene to generate multiple protein products with differing specificities and activities (reviewed in Leff et al., 1986). The functional significance of the various mdm-1 RNA species and their protein products remains to be determined. From Western blotting and immunofluorescence studies, we find that at least one of the mdm-1 proteins is located in the nucleus, while others are detected in the cytoplasm. The region of the gene which provides a nuclear localization signal has yet to be defined. We are currently carrying out experiments to characterize those punctate nuclear structures recognized by the anti-mdm-1 sera and determine if mdm-1 encodes a DNA binding protein. If mdm-1 proteins are indeed associated with DNA
or with transcription complexes, it is possible that amplification and overexpression of this gene in the 3T3DM cells could well influence cellular proliferation or gene expression.

We find that the 5' region of the mdm-1 gene has certain properties in common with those of classes of genes sometimes referred to as "growth control" genes, as well as "housekeeping" genes. These characteristics include high G+C content, absence of CCAAT and/or TATA boxes, presence of potential Spl binding sites, and multiple sites of transcriptional initiation. These genes also exhibit a low level of expression in a wide range of tissues (Bird, 1986; Dynan and Tjian, 1985; Kadonaga et al., 1986), as does mdm-1. We have also found sequence homology to mdm-1 in a range of mammalian species, including human (Cahilly-Snyder et al., 1987). Thus, mdm-1 is an evolutionarily conserved gene that most likely plays a role in general cellular metabolism and also may have a tissue-specific function in the testis.

Given the stable maintenance of DMs in the 3T3DM cells in the absence of obvious selective pressure, our working hypothesis is that the overexpression of one or more of the genes located on the DMs is directly associated with the transformed properties or altered growth control of the 3T3DM cells. With the information we have obtained on mdm-1, it is now possible to directly examine the transforming potential of this gene. Although cDNA constructs are often useful in assessing gene function in transfections, in this case it would not be an ideal initial approach because of the multiple mRNAs generated by mdm-1. We have instead isolated a cosmid clone containing an intact mdm-1 gene, which also contains an amplifiable dihydrofolate reductase marker. This construct is now being employed in transfection assays to determine if overexpression of the mdm-1 gene will result in an altered phenotype of recipient cells.

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