Increased Growth of NIH/3T3 Cells by Transfection with Human p120 Complementary DNA and Inhibition by a p120 Antisense Construct

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ABSTRACT

The human nucleolar antigen p120 was detected with an anti-p120 monoclonal antibody in most human malignant tumors but not in most resting human tissues (J. W. Freeman et al., Cancer Res., 48: 1244-1251, 1988) and has been used as a prognostic tumor marker in breast cancer patients (J. W. Freeman et al., Cancer Res., 51: 1973-1978, 1991). After the complementary DNA and gene for the human p120 protein were isolated and sequenced (review: H. Busch, Cancer Res., 50: 4830-4838, 1990), constructs were prepared to study the expression of the p120 complementary DNA (pSVX120) or the antisense, p021 DNA (pSVX021), and clones containing these constructs were selected. The expression of p120 or p021 in these constructs was regulated by Moloney murine leukemia virus long terminal repeats. In pSVX120-transfected NIH/3T3 cells, the expressed human p120 protein was localized to the nucleoli as shown by anti-p120 monoclonal antibody immunofluorescence. Expression of the p120 message and protein was confirmed by Northern (mRNA) and Western (protein) blots. Transfection of the p120 complementary DNA in sense orientation caused malignant transformation of NIH/3T3 cells in vitro and produced rapidly growing tumors in nude mice. Transfection of the antisense p120 constructs markedly delayed the growth of these tumors in vitro and in vivo (L. Perlaky et al., Proc. Am. Assoc. Cancer Res., 32: 1682, 1991). When transformed 3T3/pSVX120 cells were transfected with an inducible antisense p120 construct (pMSG021), dexamethasone induction decreased the growth rate by 62%, and the cell line returned to its normal phenotype. Northern blot analysis showed a decreased level of p120 mRNA, and the immunofluorescence was also markedly reduced.

INTRODUCTION

One of the major targets for cancer cells is their abnormal, pleomorphic nucleoli (1-3). Previous studies with MAbp120 (1, 4) have shown positive nucleolar fluorescence in pleomorphic nucleoli of many human cancers but not in most normal resting human cells (4). Additional studies indicated that the p120 is a proliferation-associated nucleolar antigen which is visualized in the early G1 phase of the cell cycle (1) and peaks in S phase (5). A recent clinical study (1, 6) showed that increased survival of breast cancer patients correlated with reduced amounts of the p120 protein and that the p120 protein is a prognostic marker in such cases. Ochs et al. (7) found by immuno-electron microscopy that the p120 protein was localized to a beaded microfibrillar network of the nucleolus and suggested that the p120 antigen is a component of the nucleolar matrix of the highly pleomorphic nucleoli of cancer cells.

Multiple overlapping cDNA clones for p120 were isolated and sequenced (8); the genomic DNA sequence was also determined (1, 9). A 2.5-kilobase upstream segment of the p120 gene was found to have important cis-acting elements at -400- and -1400-base pair regions (10). The p120 protein was shown to have basic, acidic, hydrophobic, methionine-rich and cysteine-proline-rich domains. Recently, the p120 protein was found to be phosphorylated at serine, threonine, and tyrosine residues (11). Using a series of constructs, the p120 epitope recognized by the MAbp120 was defined as an octapeptide consisting of amino acid residues 173-180 (12).

The function of the human p120 protein is not known; it may function like an oncogene (1, 13). In this study, the p120 cDNA and its antisense DNA were subcloned separately into expression vectors. These constructs provided an opportunity to analyze the effects of expression of the human nucleolar antigen p120 in NIH/3T3 cells. In addition, it was of interest to determine whether the antisense p120 constructs affected the growth of NIH/3T3 cells or p120-producing, transformed NIH/3T3p120 cells in vitro and in vivo.

MATERIALS AND METHODS

Cloning of p120 cDNA into an Expression Vector. The plasmid pET120, which contained the p120 cDNA (11), was cut with NcoI and SspI and then treated with Klenow DNA polymerase I. The two fragments were separated on 1% agarose gel, and the 3.0-kilobase p120 cDNA was excised and purified using the Gene clean kit (Bio 101, Inc.).

An expression vector, pSVX, that contains the neomycin resistance gene (14) was linearized with BamHI and blunt ended with Klenow fragment. The purified p120 cDNA fragment was ligated with the linearized pSVX. Insertion of the p120 cDNA in the BamHI site of pSVX increases p120 expression under the control of Moloney murine leukemia virus LTR (Fig. 1A). The orientation of the insert was determined by HindIII digestion. Clones containing the p120 cDNA in the sense direction are referred to as pSVX120. The "antisense" clones contain the reverse orientation of the p120 cDNA (pSVX021). The orientation is with respect to the upstream LTR of the pSVX vector. To learn more about the effect of the antisense p120, another construct was made using the pMSG vector. The full-length p120 cDNA in reverse orientation was cloned downstream of the mouse mammary tumor virus LTR and was designated as pMSG021 (Fig. 1B). These pMSG constructs have a dexamethasone-inducible gene and a gpt selection gene.

Transfection by Electroporation. Logarithmic-phase growth cells were harvested with trypsin/EDTA, centrifuged at 800 rpm for 5 min in a Fischer Centrifuge centrifuge, and washed in PBS (10 mm phosphate, 150 mm NaCl, pH 7.2); 3 × 10^6 cells/ml were resuspended in 1× 4-(2-
Fig. 1. Vector constructs containing p120 cDNA sequences. In A, the pSVX vector is described by Cepko et al. (14). The p120 cDNA was inserted downstream of the LTR, and the orientation of the p120 sequence was determined by digestion with HindIII. Two other HindIII sites are not shown. The pBR322 sequence contains the β-lactamase gene. In B, the p120 cDNA in reverse orientation (antisense) was cloned downstream of the mouse mammary tumor virus LTR (MAMTV LTR) and referred as pMSG021. LTR, Moloney murine leukemia virus long terminal repeat; ATG, p120 translational initiation codon; stop, p120 translational stop codon; 3' SS, Moloney MuLV 3' splice site; NEO, Tn5 neomycin resistance gene; SVori, simian virus 40 origin of replication; pBRori, pBR322 origin of replication.

hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.05, 137 mM NaCl, 0.5 mM KCl, 0.7 mM Na2HPO4, 6 mM dextrose) containing 500 µg/ml of sonicated salmon testis DNA (Sigma). The pSVX or pMSG plasmid constructs (20 µg/ml DNA) (Fig. 1) were then added. The cells were exposed to a single voltage pulse (220 V, 960 µF; Gene-Pulser; Bio-Rad) at room temperature, allowed to remain in the buffer for 10 min, and then plated onto 10-cm cell culture dishes (Falcon). The optimal parameters of electroporation (220 V, 960 µF, single pulse) were determined previously for NIH/3T3 cells by colony-forming assays (cell killing) and MAbp120 immunostaining (gene transfer). The sense p120 (pSVX120), the antisense p120 (pSVX021), or the pSVX vector alone were electroporated into NIH/3T3 cells. The purified DNA was precipitated by addition of 0.1 volume of 3.5 M sodium acetate and 2.5 volumes of ethanol. DNA was digested with restriction enzymes according to reaction conditions recommended by BRL-Gibco. The DNA fragments were separated on 0.8% agarose gels and transferred to Zeta-Probe blotting membrane (Bio-Rad). Blotting, prehybridization, hybridization, and washing of filters were carried out according to the manufacturer’s instructions.

NPT II Enzyme-linked Immunosorbent Assay. The presence of the pSVX constructs in the NIH/3T3 cells was further analyzed by the expression of NPT II. NPT II was detected in the transfected cells with an enzyme-linked immunosorbent assay kit (5 Prime - 3 Prime, Inc.). The various transfected cell lines that grew on 10-cm cell culture dishes were scraped and transferred to a conical tube. The pellets were suspended in 200 µl of PBS and were subjected to three freeze (at −70°C) and thaw (at 37°C) cycles, of 10–15 min each. The supernatants were collected, stored at −70°C, and analyzed for NPT II.

Protein Blots. The whole cells from transfected and nontransfected NIH/3T3 cells were solubilized in Laemmli buffer and heated at 100°C for 5 min. The extracts were loaded onto a sodium dodecyl sulfate (0.1%) polyacrylamide (7.5%) gel and electrophoresed for 1 h at 200 V on a Bio-Rad minigel apparatus. Proteins were transferred to nitrocellulose membrane by the method of Towbin et al. (16). The available binding sites were treated with blocking buffer (10 mM Tris-HCl, pH 7.5, 3% bovine serum albumin, 150 mM NaCl, 10% chicken serum). The MAbp120 was added at a 1:400 dilution of ascites in a buffer of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20 and incubated for 2 h at room temperature. The second antibody, a phosphatase-conjugated goat anti-mouse (Promega), was added at 1:5000 dilution; incubation was for 1 h. The band was developed in substrate-containing buffer (Promega); the reaction was terminated with a 20 mM Tris-HCl, pH 8.0, 2 mM EDTA buffer.

RNA Preparation and Analysis. Polyadenylated RNAs were prepared with a Fast Track mRNA isolation kit (Invitrogen Co., San Diego, CA). Equal amounts of polyadenylated RNA were denatured and fractionated on a 1.2% agarose gel containing formaldehyde (17) and transferred to Zeta-Probe blotting membrane (Bio-Rad). Sense and antisense hybridization probes were synthesized with an RNA transcription kit (Stratagene) and pBS120 template (p120DNA in the Blue-script vector; Stratagene). Prehybridization and hybridization were done as recommended by the supplier.

Indirect Immunofluorescence. Asynchronous cells in logarithmic growth phase were used for immunostaining. The cells were grown on slides, air dried, and fixed in formaldehyde/PBS for 20 min. The slides were washed in PBS, and the cells were permeabilized in acetone at −20°C for 4 min (7). Anti-p120 monoclonal (MAbp120) or polyclonal (PAbp120) antibodies (1:50 or 1:20 dilution) were added and incubated in a moist chamber at 37°C for 60 min (4); the sample was washed three times in PBS for 20 min/wash. The primary antibody was detected with fluorescein-conjugated, goat anti-mouse immunoglobulin (Cappel) (dilution, 1:20 in PBS) at 37°C for 35 min. The slides were washed three times in PBS and covered with n-propyl-gallate containing glycerol-PBS (18).

Cell Culture. NIH/3T3 cells (American Type Culture Collection CRL 1658, contact-inhibited NIH Swiss mouse embryo) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin liquid (10,000 IU/ml penicillin G sodium, 10 mg/ml streptomycin sulfate in 0.85% saline) (Gibco). Based on the doubling time and on the 3-day plating schedule, 6.5 × 105 exponentially growing cells were serially plated into T-75 cell culture flasks (Falcon). All cell lines were negative for Mycoplasma infection as determined by a DNA stain (19, 20).

Colonies Formation in Soft Agarose. The NIH/3T3 pSVX, NIH/3T3pSVX021, and NIH/3T3pSVX120 cultured clones were trypsinized, and 2.5 × 10^5 exponentially growing cells were serially plated into T-75 cell culture flasks at 37°C with 0.8% agarose base. The triplicate plates were incubated in a humidified incubator at 37°C for 3–6 weeks. The plates were stained with p-
iodonitrotetrazolium violet (Sigma), and the colonies with a diameter greater than 0.2 mm were counted under a 7× measuring magnifier (21).

In Vivo Studies in Nude Mice. The exponentially growing NIH/3T3 cells (NIH/3T3, NIH/3T3pSVX, NIH/3T3pSVX021) were washed and resuspended in serum-free DMEM. Viable cells (2 × 10⁶) (determined by trypan blue exclusion) in 0.2 ml DMEM were injected s.c. into homozygous mutant Hsd: Athymic Nude-nu male mice (22).

Tumor growth was followed by daily measurement of the three orthogonal diameters (L, W, and H), and volume (V) was calculated as \( \pi/6 \times (L \times W \times H) \) (23). All animal experimentation followed the guidelines of the Baylor College of Medicine and New York Academy of Sciences.

![Fig. 2. Southern blot analysis of the total DNA from NIH/3T3 clones and estimation of the gene copy number. Ten \( \mu \)g of total DNA were digested to completion with HindIII (A) or EcoRI (B), electrophoresed on 0.8% agarose gels, and transferred to Zeta-Probe nylon membranes (Bio-Rad). The filters were hybridized to randomly primed \( ^{32} \)P-labeled pSVX120 whole plasmid and washed according to the supplier of the membrane (Bio-Rad). A and B: Lane 1, nontransfected NIH/3T3; Lane 2, NIH/3T3pSVX (vector alone transfected); Lane 3, NIH/3T3pSVX021 (antisense p120 construct transfected); Lane 4, NIH/3T3pSVX120 (sense p120 construct transfected); Lane 5, 90 pg pSVX120 plasmid. Ordinate, size of molecular weight markers in kilobases. In C, the gene copy number was estimated by dot blot hybridization. Each standard pSVX120 plasmid, corresponding to 1–5 copies, was mixed with 10 \( \mu \)g of NIH/3T3 total DNA. The copy number was determined using 10 \( \mu \)g total DNA of each clone and probed with \( ^{32} \)P-labeled pSVX120 plasmid. The filter was cut, and the radioactivity of each dot was determined using a liquid scintillation counter.](cancerres.aacrjournals.org)
RESULTS

Presence of the pSVX Recombinant Plasmid in Transfected NIH/3T3 Cells. To determine whether the Geneticin-resistant NIH/3T3 clones contained the pSVX recombinant plasmids, Southern blot analysis, dot blot hybridization, and neomycin phosphotransferase II assays were performed. The analysis of total DNA from the transfected NIH/3T3 clones digested with restriction enzymes showed the presence of bands that hybridized with the $^{32}$P-labeled pSVX120 probe (Fig. 2, A and B). The hybridizing bands from the NIH/3T3pSVX120 clone digested with HindIII (Fig. 2A, Lane 4) or EcoRI (Fig. 2B, Lane 4) showed patterns similar to that of the pSVX120 plasmid digested with the same enzymes (Fig. 2, A and B, Lanes 5). This result implies that the plasmid was not integrated into the NIH/3T3 genome. Despite the low gene copy number (Fig. 2C), the plasmids were retained by the cells after 10 passages. The presence of the SV40 origin of replication (SVori) in the constructs (Fig. 1A) may enable them to replicate as episomes.

Total DNA from NIH/3T3pSVX and NIH/3T3pSVX021 digested with HindIII or EcoRI had a number of bands that hybridized with the $^{32}$P-labeled pSVX120 probe (Fig. 2, A and B, Lanes 2 and 3). The clones contained 1–2 copies of the construct per cell (Fig. 2C). The presence of the pSVX vector in the clones was further confirmed by the expression of the neomycin phosphotransferase II (125–355 pg NPT II/mg total protein) from the neomycin resistance gene.

Expression of pSVX120 mRNA in NIH/3T3 Cells: Northern Blots. To determine whether the transfected sense or antisense p120 constructs were expressed in NIH/3T3 cells, polyadenylated RNA was prepared from pSVX, pSVX021, and pSVX120 clones for Northern blotting. Equal amounts of polyadenylated

Probe: Sense p120 RNA

Antisense p120 RNA

Fig. 3. Northern blot analysis of the expressed sense and antisense p120 transcripts. Equal amounts of polyadenylated RNA (2.5 µg/lane) were fractionated in a 1.2% agarose gel containing formaldehyde and analyzed as described in "Materials and Methods." Sense and antisense p120 transcripts were detected using sense (A) and antisense (B) p120 riboprobes, respectively. Ordinate, size in kilobases of RNA markers.

Fig. 4. Immunofluorescence detection of antigen p120 in transfected NIH/3T3 cells. The cells were grown on slides, fixed, permeabilized, and then subjected to immunofluorescence with MAAbp120 as described in "Materials and Methods." A, representative photograph of undetectable nucleolar fluorescence in a NIH/3T3pSVX clone. The same results were observed in NIH/3T3 or NIH/3T3pSVX021 clones. $\times$ 375. In B, NIH/3T3pSVX120 clones showed bright nucleolar fluorescence. $\times$375.

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Fig. 5. Western blot analysis of nucleolar antigen p120 in transfected NIH/3T3 whole cell extract. Western transfer from a 7.5% Laemmli gel contained whole cell extract from the different clones (Lane 3, NIH/3T3pSVX; Lane 4, NIH/3T3pSVX021; Lanes 5 and 6, NIH/3T3pSVX120 different concentration) and HeLa nucleolar extract (Lane 1) boiled in Laemmli buffer. The blot was developed with MAbp120 and the Promega phosphatase reagents. The prestained markers (Lane 2) show 6 bands of the following molecular weights: 180,000, 116,000, 84,000, 58,000, 48,500, and 36,500. Arrow, p120 band.

RNA were fractionated on agarose gel and hybridized to a 32P-labeled p120 riboprobe (Fig. 3). The sense transcript (7.5 kilobases, the length between the two LTRs containing p120 cDNA) was detected in the RNA from pSVX120-transfected NIH/3T3 cells (Fig. 3B, Lane 3) but not in the RNA from pSVX- or pSVX021-transfected NIH/3T3 cells (Fig. 3B, Lanes 1 and 2). Antisense transcripts were detected in the pSVX021 clone (Fig. 3A, Lane 2) but not in the pSVX- or pSVX120-transfected NIH/3T3 cells. The shorter transcript (6.5 kilobases) probably represents a spliced transcript. The 2.8-kilobase band detected by the p120 antisense riboprobe in the three samples (Fig. 3B) probably represents mouse p120 mRNA, which is similar in size to the p120 mRNA from HeLa cells.

Immunocchemical Detection of Antigen p120. Cells grown on slides were fixed, permeabilized, and analyzed by indirect immunofluorescence using MAbp120. The NIH/3T3pSVX120 clones exhibited bright nucleolar fluorescence (Fig. 4B), which indicated the presence of the human p120 protein. There was no detectable fluorescence in the nontransfected NIH/3T3, NIH/3T3pSVX, and NIH/3T3pSVX021 clones (Fig. 4A), because the MAbp120 is human specific and does not immunoreact with mouse nucleolar proteins.

Fig. 5 shows the results from Western blot analysis using specific MAbp120. Lane 1 shows a positive control with HeLa nucleoli; p120 is the major band. Lane 2 contained the pretranslational molecular weight markers; the Mf, 116,000 marker was juxtaposed to the p120 band in the HeLa extract. Lanes 3 and 4, which did not contain p120, were whole cell extracts from the NIH/3T3pSVX clone and the NIH/3T3pSVX021 clone, respectively. Lanes 5 and 6 contained whole cell extracts from the NIH/3T3pSVX120 clone; the p120 bands were clearly seen (arrow).

Growth in Complete or Serum-free Medium. In complete medium, the NIH/3T3, NIH/3T3pSVX, or NIH/3T3pSVX120 cells grew at similar rates; the PDT were not significantly different. Approximately 24 h were required for confluency. The NIH/3T3 and NIH/3T3pSVX cells were contact inhibited by the 6th day after plating. The NIH/3T3pSVX120 transfected clone started to form multiple layers, overgrew from the 5th day after plating, and formed rapidly growing foci. The PDT for this multiple-layered overgrowing phase was 106 h. The antisense p120 construct-containing NIH/3T3pSVX021 cells were contact inhibited by day 12 and grew more slowly than the control; the PDT was 40 h (Fig. 6A). Although the NIH/3T3pSVX120 cells did not require serum for growth, their growth in serum-free medium was slower than in serum-containing medium; the PDT was 115 h, which is similar to the PDT of NIH/3T3pSVX120 in the overgrowing phase in complete medium (Fig. 6B). The NIH/3T3pSVX and NIH/3T3pSVX021 clones divided only once or twice but no further in the serum-free medium. The NIH/3T3 cells without serum died during the 2-week period.

Growth on Confluent Monolayers. Colony formation was observed when the NIH/3T3pSVX120-transformed cells were plated on top of the contact-inhibited NIH/3T3 monolayer (Fig. 7B). The colony-forming efficiency was 20%. The NIH/3T3, NIH/3T3pSVX, or NIH/3T3pSVX021 cells showed no colony formation above the confluent NIH/3T3 monolayer (Fig. 7A), which indicates their requirement for anchorage-dependent growth.

Growth in Soft Agarose. In these studies, transfected NIH/3T3 cells were plated in soft agarose. In three repetitive experiments, 1,000, 5,000, and 10,000 cells were seeded into three parallel wells. Only the NIH/3T3pSVX120-transformed cells formed colonies that grew progressively to larger than 0.2 mm
Fig. 7. Growth of transfected NIH/3T3 clones on top of the contact-inhibited NIH/3T3 monolayer. A, representative result of NIH/3T3, NIH/3T3pSVX, or NIH/3T3pSVX021 cells showing no colony formation on top of the confluent, contact-inhibited NIH/3T3 monolayer. × 90. B, intensively growing colony of NIH/3T3pSVX120-transformed cells on top of the confluent, contact-inhibited NIH/3T3 monolayer. × 90.

Fig. 8. Growth of transfected NIH/3T3 cells in soft agarose. A, representative plate of NIH/3T3pSVX cells showing no soft agarose colony formation. × 37.5. Lack of colony formation was also observed with NIH/3T3 and NIH/3T3pSVX021 cells. B, soft agarose colonies of NIH/3T3pSVX120-transformed cells. × 37.5.
Fig. 9. Tumor growth in nude mice. Transfected NIH/3T3 cells were transplanted s.c. into nude mice as described in "Materials and Methods." Triplicate experiments were made at different times, using different batches of clones. Three to six animals were used in a group for each experiment. Data from a representative experiment are shown. The NIH/3T3pSVX120 cell-induced tumors grew faster than the NIH/3T3pSVX-induced tumors. The NIH/3T3pSVX021 (antisense pl20)-induced tumors grew much more slowly than either the NIH/3T3pSVX (vector alone)- or NIH/3T3pSVX120 (sense pl20)-induced tumors; the difference in growth was significant (P < 0.01).

diameter in soft agarose (Fig. 8B). The colony-forming efficiency of the pl20-containing NIH/3T3 cells was 5.6%. The NIH/3T3, NIH/3T3pSVX, and NIH/3T3pSVX021 cells did not form colonies in semisolid medium (colony-forming efficiency, 0.05%) (Fig. 8A).

Tumor Growth in Nude Mice. Preliminary studies were begun to assess the in vivo growth characteristics of the three transfected cell lines. In these studies, transfected NIH/3T3 cells were transplanted s.c. into nude mice. Experiments were done in triplicate, using three different batches of each transfected cell line. In each experiment, three or six animals were used in a group. Fig. 9 shows that the NIH/3T3pSVX120 cells induced tumors which grew more rapidly than the NIH/3T3pSVX-induced tumors. The NIH/3T3pSVX021 (antisense induced tumors) which grew very slowly; their growth delay was much longer than those of the NIH/3T3pSVX- or NIH/3T3pSVX120-induced tumors. The parameters of tumor growth (Table 1) show that the tumor growth delay was 12.3 days for the cells containing the antisense construct and 6.0–6.5 days for the pSVX or pSVX120 construct-containing tumors. The tumor growth time for the antisense pSVX021-containing tumor was 4–6 times greater than for the pSVX or the pSVX120 tumors.

Effect of Antisense pl20 Constructs on Transformed NIH/3T3pSVX120 Cells. To learn more about the effect of the antisense pl20, the previously characterized, transformed NIH/3T3pSVX120 cells producing human pl20 were transfected by electroporation with the antisense, pMSG021 construct. Fig. 10 shows the growth characteristics of the transformed sense NIH/3T3pSVX120 cells transfected with the pMSG vector alone (circles) and the transformed clones after transfection with the antisense pl20 construct (triangles). The growth rate (\(G = \ln 2/DT\)) of the sense, NIH/3T3pSVX120 transfected with the antisense, pMSG021 (open triangles, solid line, \(G = 1.0 \times 10^{-2} \text{ h}^{-1}\)) was reduced by 52% compared to the sense NIH/3T3pSVX120 transfected with the pMSG vector alone (open circles, solid line; \(G = 2.1 \times 10^{-2} \text{ h}^{-1}\)). After stimulation with dexamethasone, the growth rate was reduced by 62% (dashed line with filled triangles; \(G = 7.7 \times 10^{-3} \text{ h}^{-1}\)). Northern blot analysis showed a decreased level of pl20 mRNA, the immunofluorescence was also markedly reduced, and the cells returned to their original untransformed phenotype (data not shown).

**DISCUSSION**

Several constructs were prepared using the pSVX vector for transfection into NIH/3T3 cells; these constructs contained the complete pl20 cDNA in sense (forward; pSVX120) and anti-

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<th>Clone</th>
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<th>TVDT-2 (h)</th>
<th>TGT (days)</th>
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<td>154</td>
<td>68.9</td>
<td>1.4 x 10^{-2}</td>
<td>4.5 x 10^{-3}</td>
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* TGD, tumor growth delay (time from s.c. transplantation of cells until the tumor reached 10 mm³ volume); TVDT-1, tumor volume doubling time (calculated from the first phase of log/linear plot of growth curves); TVDT-2, tumor volume doubling time (calculated from the second phase of log/linear plot of growth curves); TGT, tumor growth time (time of tumor growth from 10 mm³ until 1000 mm³); TG, tumor growth rate (calculated as ln2/TVDT); TG-1, tumor growth rate of the first phase of tumor growth; TG-2, tumor growth rate of the second phase of tumor growth.
sense (reverse; pSVX021) orientations with respect to the LTR. Following electroporation into NIH/3T3 cells and selection of the clones, 1–2 copies of the plasmids were present per cell. Northern blots using labeled p120 riboprobes indicated that the sense p120 and the antisense p021 transcripts were produced. The presence of p120 and p021 mRNA was further confirmed by RNase protection assay (data not shown). Cells containing the cDNA in the sense orientation produced human p120, which localized to the nucleolus as shown by indirect immunofluorescence; the p120 protein was also shown by Western blot analysis to be present in whole-cell extracts.

Frequently, transformed cells have lower serum dependence than their normal counterparts (24). Their properties are associated with in vitro transformation and are related to changes in growth characteristics, genetic properties, and neoplastic properties (25). The anchorage-independent growth of NIH/3T3/pSVX120 cells and their cytomorphical changes are characteristic of a transformed phenotype, suggesting that the p120 constructs might function like an oncogene. Similar anchorage- and serum-independent growth was found in NIH/3T3 cells transfected with the ras oncogene (26, 27) or the hcc65 oncogene derived from a human hepatocellular carcinoma (28).

The transfected p120 cDNA in the sense orientation resulted in the loss of contact inhibition in monolayers and colony formation in soft agarose. Neither the control pSVX vector nor the antisense pSVX021 produced these effects.

In vivo studies on Hsd:Athymic Nude-nu male mice showed that the cells transfected with p120 in the sense orientation produced rapidly growing solid tumors. These tumors were visible 1 week following the s.c. transplantation. Nontransfected NIH/3T3 cells (27) or transfected NIH/3T3 cells with the vector alone produced tumors that grew more slowly.

The presence of the antisense, pSVX021 construct in NIH/3T3 cells markedly delayed tumor growth when compared with the vector alone and with the p120 in the sense orientation. The slower growth of the antisense p120-containing NIH/3T3 cells (NIH/3T3/pSVX021) (Figs. 6 and 9) may result from effects on the NIH/3T3 mouse p120 mRNA. Preliminary experiments in our laboratory have shown a 77% nucleotide similarity between the human and mouse p120 cDNA. Although there have been many reports on the use of antisense molecules to affect gene expression (29, 30), this study provides evidence that the whole antisense construct reduced the growth rate of these cells in vivo.

The growth of p120-containing cells was markedly inhibited by transfection of the antisense p120 construct (pMSG021) and was inhibited even more by dexamethasone stimulation.

The mechanism of the increased growth rate of the tumors and cells transformed by the p120 sense construct is not clear. The overproduction of the p120 protein may activate other genes or accelerate other cellular growth events. The growth-inhibitory effect of the antisense construct is particularly interesting and may lead to the use of antisense oligonucleotides in cancer treatment.

Oligonucleotides designed to hybridize to specific mRNA sequences have been utilized to inhibit the expression of specific proteins. Antisense oligonucleotides have been used successfully to inhibit oncogenes such as c-myc or c-myb (31, 32). Fonagy et al. (5) demonstrated inhibition of p120 protein expression and cell proliferation with an antisense oligonucleotide in a human lymphocyte system in vitro.

Since the sense p120 protein increased cell proliferation and malignant transformation of normal NIH/3T3 cells, and the antisense p120 inhibited the increased cell growth and returned the p120-transformed cell line to its normal phenotype after dexamethasone induction, antisense p120 oligonucleotide molecules appear to have potential value as therapeutic anticancer agents. The possibility that either the antisense cDNA or specific antisense sequences may have therapeutic use is currently under further study.

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