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Rapid and Selective Alterations in the Expression of Cellular Genes Accompany Conditional Transcription of Ha-v-*ras* in NIH 3T3 Cells

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Hormone treatment of NIH 3T3 cells that contain recombinant fusions between the mouse mammary virus long terminal repeat and the v-*ras* gene of Harvey murine sarcoma virus results in conditional expression of the *ras* p21 gene product. Levels of *ras* mRNA and p21 are maximal after 2 to 4 h of hormone treatment. Analysis of cellular RNA by Northern blotting and nuclease S1 protection assays indicates that the expression of two cellular RNA species increases with kinetics similar to v-*ras*: v-*sis*-related RNA and retrovirus-related VL30 RNA. Run-on transcription in isolated nuclei shows that the increase in v-*sis*-related RNA is not dependent on transcription and therefore must arise by a post-transcriptional mechanism. The increase in VL30 expression is a transcriptional effect. Hormone treatment of normal NIH 3T3 cells has no effect on the expression of these DNA sequences. These results suggest that v-*ras* stimulation of autocrine factors may play a role in transformation of cells by this gene and also suggest a reverse genetic strategy to determine the nucleic acid sequences and cellular factors involved in the regulation of gene expression that is observed.

The effect of oncogenes on cellular gene expression and the role of such effects in malignant transformation remain intriguing questions. One attractive possibility is that oncogenes may function, at least in part, by stimulating (or repressing) the expression of key regulatory genes. This mechanism of action is especially pertinent for oncogenes whose protein products are located in the nucleus, such as *myb*, *myc*, *fos*, and the E1A gene of adenoviruses (4).

However, even oncogene protein products whose primary location, and hence their assumed primary action, are nonnuclear can affect the expression of cellular genes. For example, chicken embryonic cells transformed by the plasma membrane-localized oncogene v-*src* express on the order of 1,000 genes that were not active before transformation (22).

In addition, the identity of certain oncogenes with growth factors or growth factor receptors (13, 47) indicates that oncogene proteins associated with the cell plasma membrane may influence gene expression. For example, the v-*erb*, B-oncogene product is related to the plasma membrane receptor for epidermal growth factor (13). It has been clearly demonstrated that epidermal growth factor can rapidly and selectively alter the expression of genes in target cells (16, 20, 33).

One family of oncogene proteins whose primary location is the inner surface of the plasma membrane is the *ras* gene family (14). Several lines of evidence suggest that transformation by *ras* genes may cause alterations in the expression of cellular gene products. First, infection of cells with either Harvey sarcoma virus or Kirsten sarcoma virus has been correlated to the production of a platelet-derived growth factor (PDGF)-like activity and down regulation of PDGF receptors (6). Second, cells transformed by Harvey sarcoma virus produce 20 to 50 times more transforming growth factor beta than nontransformed cells do (1). Third, DeLarco and co-workers (10) observed that the production of sarcoma virus growth factors by cells transformed by a temperature-sensitive mutant strain of Kirsten sarcoma virus is shut off at the nonpermissive temperature. These authors also demonstrated that the sarcoma virus growth factors were not the thermolabile molecules. Fourth, transformation of cells by the microinjection of purified, mutant *ras* protein is blocked if either RNA or protein synthesis is inhibited. One interpretation of these results is that the de novo synthesis of RNA or proteins or both is required for *ras* transformation (15).

By putting the Ha-v-*ras* gene under the control of the hormone-modulated mouse mammary tumor virus (MMTV) promoter, we have been able to regulate *ras* expression in NIH 3T3 cells in an inducible fashion: if steroid hormone is added to the cell culture medium of cells containing these chimeric molecules, the cells are transformed, while removal of the hormone inducer causes a reversion of the transformed phenotype (23). Therefore, these cells provide a way to examine whether stimulation of v-*ras* expression results in the alteration of cellular gene expression. In particular, this system allows questions concerning the mechanisms of action involved to be rigorously addressed in a temporal fashion with respect to v-*ras* expression.

In the present work, we have analyzed the expression of a few cellular genes by monitoring both steady-state RNA levels and nuclear transcription rates. We find two genes whose expression is affected when concentrations of the *ras* gene product increase intracellularly: a v-*sis*-related gene, and a family of retrovirus-like DNA sequences termed VL30 elements (8, 28). Further, our analysis indicates that regulation of the former is post-transcriptional in nature, while regulation of the latter sequences involves primarily a transcriptional mechanism.

MATERIALS AND METHODS

Growth and hormone treatment of cells. NIH 3T3 and 433 (25) cells were cultured in Dulbecco modified Eagle medium supplemented with 10 and 5% fetal bovine serum (GIBCO Laboratories), respectively. Cells were kept for 48 to 72 h past the point when they had reached confluency. Treatment of cells at this point with tritiated thymidine indicated that DNA synthesis had ceased. At this point, 10^{-7} M dexamethasone was added without changing the medium; control cultures received no hormone. Hormone treatment was for

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differing lengths of time (see figure legends). In some experiments, as noted, Opti-MEM medium (GIBCO) supplemented with 4% fetal bovine serum was used. For serum stimulation experiments, cells were treated exactly as above, but then had the medium removed and replaced with fresh medium containing 15% fetal bovine serum.

Initiation of DNA synthesis was monitored by thymidine incorporation as previously described (37).

DNA probes. The following DNA fragments were used as probes in the experiments described below: for v-*sis*, a 800-base pair (bp) *Pst*I-*Xba*I fragment isolated from simian sarcoma virus (38); c-*myc*, a 900-bp *Xba*I-*Sac*I fragment of the mouse gene that contains all of exon II (42); c-*fos*, a 1,900-bp *Eco*RI-*Sal*I fragment of the mouse gene that is missing exon I (32); v-*src*, a 840-bp *Pvu*II fragment derived from Rous sarcoma virus (Schmidt-Ruppen strain; 11); NVL-3, a 500-bp *Bam*HI-*Eco*RI long terminal repeat (LTR)-specific fragment (7); and for v-*ras*, a 600-bp *Hin*dIII-*Pst*I fragment derived from Harvey sarcoma virus (12).

For run-on transcription analysis, these probes were cloned into M13 vectors so that the antisense (−) DNA strand is obtained when the single-strand virus is isolated. In the case of v-*ras*, the sense (+) strand orientation was also constructed.

Isolation of nuclei. Cells were washed with Hanks buffered salt solution, collected by scraping with a rubber policeman, and pelleted by centrifugation. Cells were lysed by suspension at 10^7 cells per ml in a solution containing 10 mM Tris acetate (pH 7.4), 10 mM NaCl, 60 mM KCl, 3 mM $MgCl_2$, and 0.5% (vol/vol) Nonidet P-40 (lysis buffer). The nuclei were pelleted by centrifugation, washed with the lysis buffer, and suspended at 10^8 cells per ml in a solution consisting of 50 mM Tris acetate (pH 8.3), 40% (vol/vol) glycerol, 5 mM $MgCl_2$, and 0.1 mM disodium EDTA (freezedown buffer). Nuclei were stored in freezedown buffer at −70°C.

Synthesis and analysis of run-on transcripts. Run-on transcription assays were performed essentially as previously described (21). Briefly, 100 μl of nuclei prepared as described above was thawed on ice and had the following added to them: 50 μl of a buffer containing 20 mM Tris acetate (pH 8.0), 10 mM $MgCl_2$, and 100 mM KCl, 37 μl of water, 1 μl each of 0.1 M ATP, CTP, and GTP, and 10 μl of [^{32}P]UTP (10 mCi/ml; 410 Ci/mmol; Amersham Corp.). This sample was incubated for 30 min at 30°C. The reaction was terminated by the addition of RNase-free DNase I (Promega Biotech). The mixture was subjected to digestion with proteinase K (Boehringer Mannheim Biochemicals) and then extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1) and once with chloroform-isoamyl alcohol (24:1). Samples were precipitated with ethanol, trichloroacetic acid, and ethanol, in succession. Each sample was then suspended in 500 μl of a solution containing 5× SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, 0.2% bovine serum albumin, 0.2% polyvinylpryrrolidone (Sigma Chemical Co.), 0.2% Ficoll 400 (Pharmacia) and 50% (vol/vol) formamide (hybridization buffer) and counted by liquid scintillation. They were then diluted to a specific activity of 5×10^6 cpm/ml in hybridization buffer plus dextran sulfate (final concentration, 10%) and hybridized with DNA probes which had been immobilized on nitrocellulose filters by slot blotting (Minifold II; Schleicher & Schuell). The DNA probes used are described above. Hybridization was for 72 h at 42°C.

After hybridization, filters were washed with 2× SSC–0.1% sodium dodecyl sulfate at room temperature versus four changes of 5 min each and then with 0.2× SSC–0.1% sodium dodecyl sulfate at 45°C versus two changes of 15 min each. Filters were exposed to Kodak XAR-5 film at −70°C with DuPont intensifying screens.

Quantitation of run-on transcription data. After autoradiography, [^{32}P]RNA was removed from individual sections of the hybridized nitrocellulose with 100 μl of 200 mM NaOH per slot. The mixture was neutralized with 100 μl of 200 mM HCl per slot. Samples were then counted by liquid scintillation.

Two normalization factors were applied to this raw data. First, ^{32}P counts found in the M13 control filter strips were subtracted. This usually amounted to the background of the liquid scintillation counter (30 dpm). Second, efficiency of hybridization in the different samples was determined, and the samples were normalized to the same efficiency of hybridization (36). To determine the efficiency of hybridization for each filter strip, ^{3}H-labeled M13 DNA (36) at constant concentration was included in each filter hybridization. The number of ^{3}H counts hybridizing to the M13 DNA probe in each sample was an indication of the relative efficiency of hybridization. In the experiments presented here, the efficiency of hybridization varied between 10 and 16%. The best explanation for this variability is that the run-on RNA samples contain different amounts of protein contamination, since use of higher concentrations of proteinase K reduced the degree of variation.

Isolation and analysis of RNA. Whole-cell RNA was isolated by guandine thiocyanate extraction as follows. Cells were washed with Hanks buffered salt solution and lysed as a monolayer by a solution containing 4 M guanidine thiocyanate, 0.1 M Tris acetate (pH 7.4), and 2 M β-mercaptoethanol. Nucleic acids were precipitated with 0.5 volume of ethanol and then suspended in a solution consisting of 5 M guanidine hydrochloride, 25 mM NaOAc (pH 5), and 143 mM β-mercaptoethanol. After precipitation with 0.5 volume of ethanol, the samples were suspended in 0.1 M sodium acetate–20 mM EDTA, pH 7. Nucleic acid was precipitated by addition of 1.5 volumes of 5 M sodium acetate, pH 5. This procedure was repeated. RNA was next suspended in water and precipitated with 2 volumes of ethanol. Finally, samples were suspended in water and stored at −70°C.

S1 nuclease protection of 5′-^{32}P-labeled DNA probe was performed by a modification of the method of Berk and Sharp (3). A 10- to 20-μg portion of RNA was coprecipitated with 50,000 cpm of 5′-^{32}P-labeled DNA probe. Samples were suspended in 80% (vol/vol) formamide–40 mM PIPES [piperazine-*N*,*N*′-bis (2-ethanesulfonic acid)] pH 6.4–0.4 M NaCl–1 mM disodium EDTA (S1 hybridization buffer), heat denatured for 10 min at 85°C, and hybridized overnight at 50°C. After incubation wtih nuclease S1 (30 U; Boehringer Mannheim) for 15 to 30 min at 37°C, the samples were extracted with chloroform-isoamyl alcohol (24:1) and ethanol precipitated. The samples were then suspended in formamide gel loading buffer and electrophoresed through a denaturing 5% polyacrylamide gel at 10 to 15 W for approximately 3 h. Gels were fixed in 20% acetic acid and 10% ethanol in water, vacuum dried onto filter paper, and autoradiographed as described above.

Poly(A) RNA selected by oligo(dT)-cellulose chromatography and analyzed by Northern blot analysis (44).

Other methods. Standard methods were used to subclone DNA sequences used as probes into M13 vectors and to isolate single-strand viral DNA (31). Western blotting was performed as described previously (45). Radioactive DNA probes were prepared by end labeling with polynucleotide

kinase for the S1 analysis or by nick translation for Northern blot analysis (31).

RESULTS

Inducible expression of Ha-v-*ras* in NIH 3T3 cells. In previously published experiments, we have demonstrated inducible expression of v-*ras* mRNA and of v-*ras* p21 protein in NIH 3T3 cells by fusing the coding sequences of the Ha-v-*ras* oncogene to the glucocorticoid-responsive MMTV promoter (25). At the outset of the present work, we wanted to scrutinize more closely the kinetics of v-*ras* induction in these cells, at both the RNA and protein levels. The cell line termed 433 (25) was used for this analysis. This cell line contains about 10 copies of the MMTV-v-*ras* construction (25). For this and all subsequent experiments, synchronized, confluent cultures of cells were used (see Materials and Methods).

S1 mapping with 5'-end-labeled probes (3) was used to measure steady-state *ras* RNA levels. For these experiments, a 227-bp restriction fragment derived from the MMTV-v-*ras* recombinant was end labeled at a unique *Bam*HI site predicted to be 117 bp downstream of the RNA cap site; this probe is specific for genes introduced via transfection (35). RNA isolated from 433 cells that were treated for increasing amounts of time with dexamethasone was hybridized to this probe. Figure 1 contains the results of the S1 mapping experiments. These experiments demonstrate the following: (i) the major S1-resistant band migrates at 118 bp, in good agreement with the predicted value; (ii) v-*ras* RNA levels achieve maximum concentration 2 to 4 h after hormone stimulation of transcription (Fig. 1A), but after 15 h of stimulation these levels have sharply decreased (Fig. 1B). Densitometric scanning of the autoradiograms indicates a 17-fold increase in RNA levels 4 h after hormone treatment and a 4-fold increase after 15 h.

Western blotting (45) of whole-cell 433 extracts utilizing a *ras*-specific monoclonal antibody (17) was used to quantitate levels of the p21 protein after various periods of hormone stimulation. Figure 2 shows that v-*ras* protein concentrations increase with the same kinetics as the mRNA. *ras* protein levels are maximal after 4 h of hormone treatment. On the other hand, dexamethasone treatment has no effect on *ras* expression in normal NIH 3T3 cells (Fig. 2, lanes 3 to 7). Additional experiments indicate that *ras* p21 concentrations remain at the steady state after 8 h of hormone treatment (data not shown); however, we have not followed p21 levels for longer than that time.

By pulsing with radioactive thymidine at different times following hormone treatment of 433 cells, we find that DNA synthesis is initiated 14 to 16 h after addition of hormone (data not shown). The same kinetics are observed for serum stimulation of these cells; the amount of thymidine incorporation is also identical for either hormone or serum stimulation of 433 cells (data not shown).

Analysis of cellular RNA in conditional *ras* cells. As the next step in our investigation, we wished to determine if the expression of any cellular genes were affected as *ras* levels increased after hormone treatment of 433 cells.

Four oncogene and proto-oncogene probes were used in these studies. These were subclones of v-*sis*, v-*src*, c-*myc*, and c-*fos* (see Materials and Methods for details). In addition, a clone of a retrovirus-like sequence termed NVL-3 was also used. NLV-3 is a clone of the so-called VL30 mouse sequences (7). We decided to use this probe because expression of such sequences has been reported to be elevated in transformed mouse cells relative to untransformed cells (9, 43).

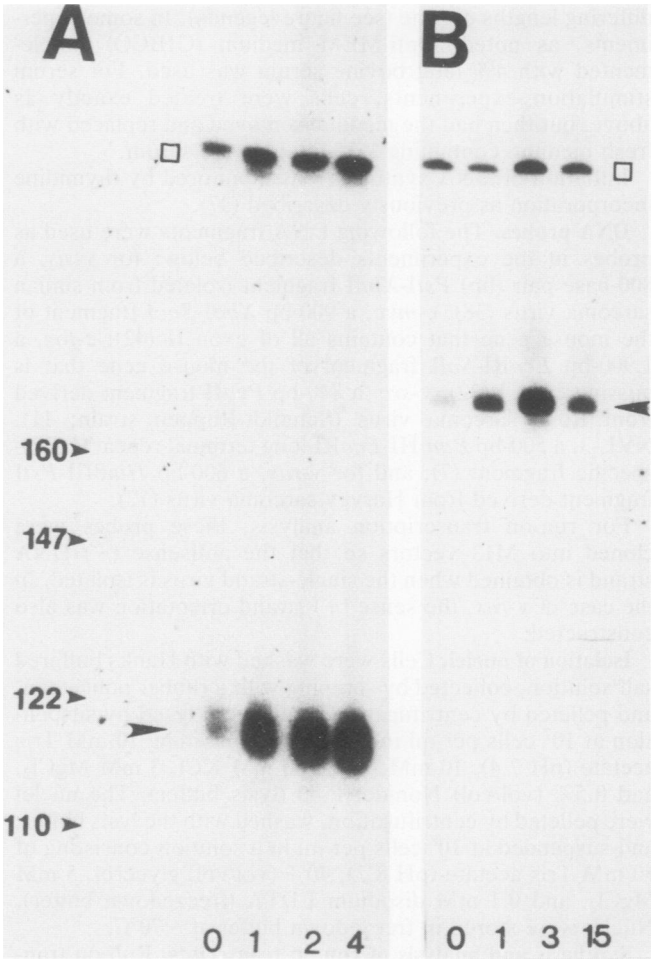

FIG. 1. S1 mapping of MMTV-initiated transcripts in 433 cells. RNA was isolated from untreated cells or from cells treated with 10^{-7} M dexamethasone. Numbers at the bottom of the lanes indicate the length of hormone treatment in hours. Small arrows in (A) indicate the position of *Msp*I-pBR322 fragments after electrophoresis. Large arrows reveal the position of the major protected band (118 bp), marking the RNA cap site. The open box shows the position of undigested probe. Exposure of autoradiograms was for 5 (A) and 3 (B) h.

Northern blotting (44) was used to analyze RNA samples with the four oncogene probes used. However, for NVL-3, an S1 protection assay was used because the NVL-3 probe is a cDNA clone obtained from cells transformed with *ras* (7) and the VL30 elements are a multicopy gene family that show a high degree of sequence heterogeneity, especially within LTR sequences (7, 27, 34). Therefore, S1 mapping may be used to measure a single member of this gene family that is known to be expressed in *ras*-transformed cells.

Representative results of the analysis of steady-state RNA by Northern blotting are shown in Fig. 3. These data show the following. (i) Within 2 h after induction of v-*ras* transcription in 433 cells, v-*sis*-related cellular mRNA increases 12-fold (Fig. 3A). After 8 h of hormone treatment, v-*sis*-related mRNA is still detectable, but the amount of this RNA present has decreased about fivefold compared with the amount present after 2 h of hormone induction. (ii) Steady-state levels for c-*myc* mRNA are unaffected after 8 h of

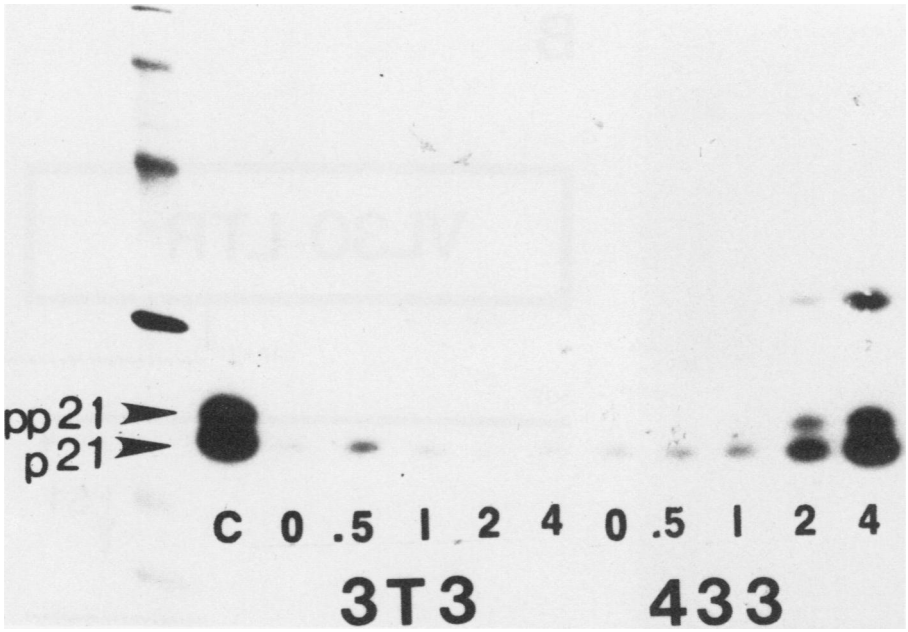

FIG. 2. Western blot of a denaturing sodium dodecyl sulfate gel containing total cellular proteins isolated from 3T3 (left) or 433 (right) cells. Blots were probed with a *ras*-specific monoclonal antibody that was labeled with ^{125}I. Numbers along the bottom of the samples indicate that the time, in hours, that cells were treated with 10^{-7} M dexamethasone before protein samples were prepared. Lane labeled C contains a protein sample isolated from cells transformed by Harvey murine sarcoma virus. Far left lane contains ^{14}C-labeled protein standards. Arrows mark the position of the phosphorylated (pp21) and unphosphorylated (p21) v-*ras* protein (14).

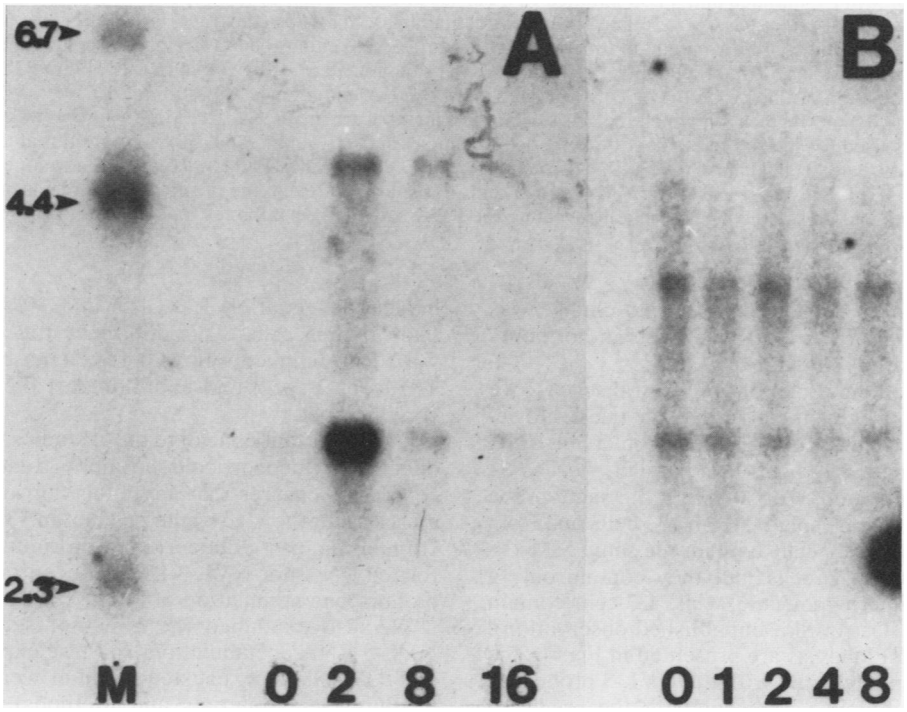

FIG. 3. Autoradiograms of Northern blots of 433 poly(A) RNA fractionated on denaturing agarose gels. Numbers at the bottom indicate the time, in hours, that cells were treated with 10^{-7} M dexamethasone before RNA was isolated. The blot in (A) was hybridized to a nick-translated v-*sis* probe, while that in (B) was hybridized to a c-*myc* probe. Lane M in (A) contained end-labeled DNA markers whose sizes were indicated in kilobases. The position of these markers apply to (A) only. Exposure of autoradiograms was for 16 h.

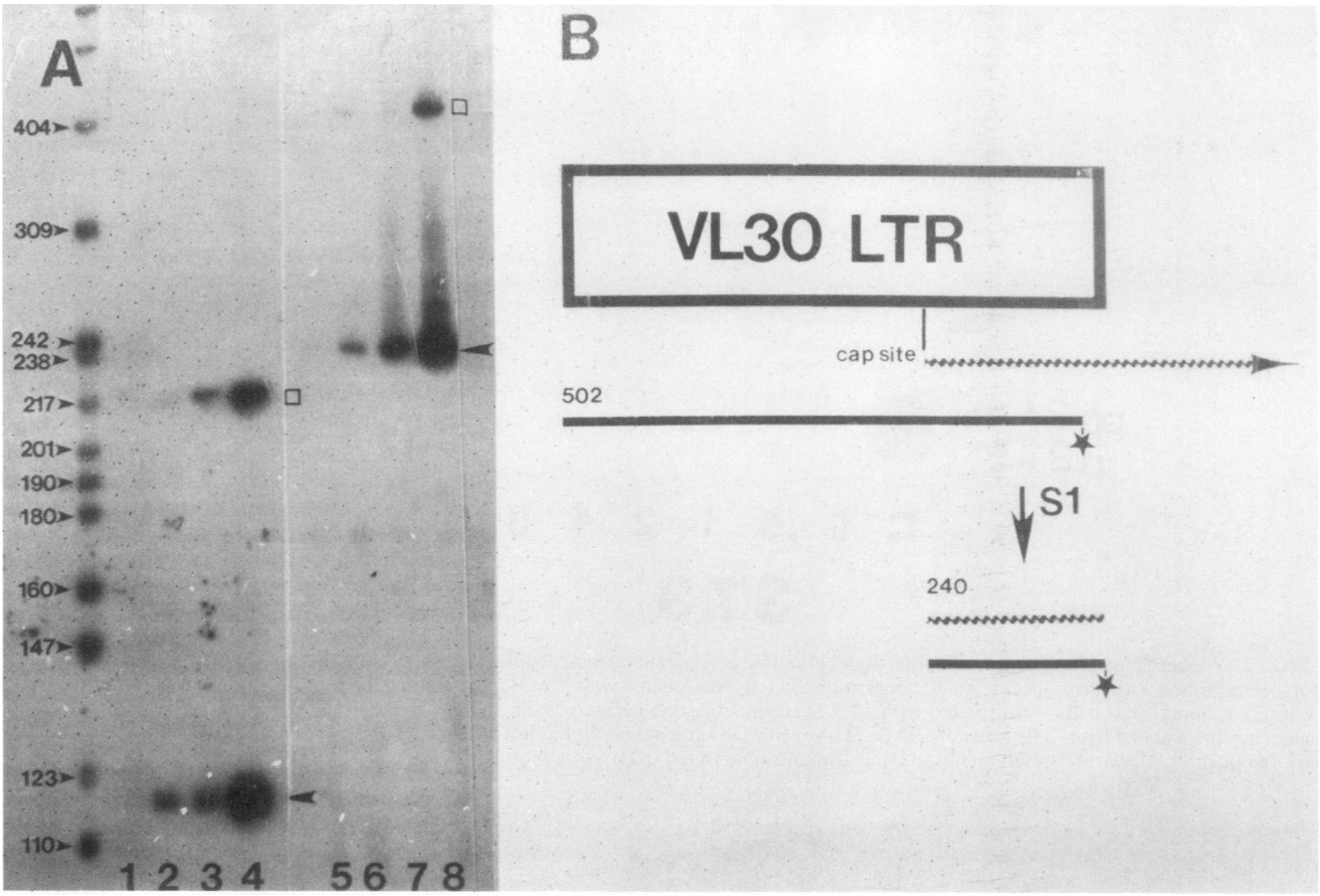

FIG. 4. S1 mapping of MMTV-initiated and VL30-initiated RNA in 433 and 1505 cells. In this experiment, cells were grown in Opti-MEM medium containing 4% fetal calf serum. RNA was isolated from cells containing MMTV-*ras* constructs either after 3 h of dexamethasone (10^{-7} M) treatment or with no treatment. A 10-μg amount of total RNA was hybridized to either the MMTV probe used in Fig. 1 or the VL30 probe shown in (B). The latter probe was a 502-bp fragment labeled at an *Eco*RI site indicated by the star in (B). A 240-bp RNA-DNA hybrid would be generated after hybridization of this probe to correctly initiated NVL-3 RNA and after S1 digestion of the hybrid (34). Shown in (A) are the autoradiograms of the denaturing gels on which the S1-resistant fragments were analyzed. The MMTV probe was used for lanes 1 to 4, and the VL30 probe was used for lanes 5 to 8. Identity of the RNA samples: lanes 1 and 5, 1505 RNA from untreated cells; lanes 2 and 6, 1505 RNA from hormone-treated cells; lanes 3 and 7, RNA from untreated 433 cells; lanes 4 and 8, RNA from hormone-stimulated cells. The unmarked lane contains *Msp*I-digested pBR322 DNA as size markers. Large arrows reveal the position of the major protected band (118 bp for the MMTV probe and 240 bp for the VL30 probe), revealing the RNA cap site. Open boxes indicate the migration of the probe band. Exposure of autoradiograms was for 5 h.

hormone treatment in 433 cells (Fig. 3C). No change was detected in either c-*fos* or c-*src* mRNA levels in hormone-treated 433 cells (data not shown).

For S1 mapping of the 5′ ends of NVL-3 transcripts, we used a 502-bp DNA fragment derived from the LTR sequences as a probe. This probe should protect an RNA fragment 240 bp in length (see Fig. 4). In parallel, we also S1 mapped the MMTV-initiated *ras* RNA with the same probe described above. RNA was isolated from 433 cells and from a cell line termed 1505. 1505 cells contain the same MMTV-*ras* recombinant as the 433 cells, but they contain only a single copy of this chimeric molecule, while 433 cells contain at least 10 copies (M. Ostrowski, unpublished observation).

The results of this S1 analysis are presented in Fig. 4. The major S1-resistant band detected with the NVL-3 probe was measured to be 240 bp, in agreement with the predicted value (Fig. 4A, lanes 5 to 8). Densitometric scanning of the autoradiograms revealed that, after 4 h of hormone treatment, NVL-3 RNA is increased fourfold in 433 cells and eightfold in 1505 cells. This analysis also revealed that the induced levels of NVL-3 RNA in 433 cells is 20-fold higher than in 1505 cells. This difference reflects the variance in v-*ras* RNA concentrations between these two cell lines (Fig. 4A, lanes 1 to 4) and approximates the difference in gene copy number.

One important control to these studies was to ascertain the effects of glucocorticoid hormone treatment of NIH 3T3 cells. This ensures that any effects observed correlate with increases in the v-*ras* gene product in 433 cells and not with stimulation by glucocorticoid hormones. Neither v-*sis*-related RNA nor NVL-3 RNA concentrations were affected by hormone stimulation of NIH 3T3 cells (data not shown).

We also examined the effect of serum stimulation on NVL-3 RNA accumulation. In these experiments, NIH 3T3 and 433 cells in exhausted medium were exposed to fresh medium (see Materials and Methods), and NVL-3 RNA concentrations were measured by the S1 assay. Figure 5 shows the results of this analysis. Densitometric scanning of these autoradiograms revealed that serum stimulation caused a 1.8-fold increase in NVL-3 RNA after 4 h, while the

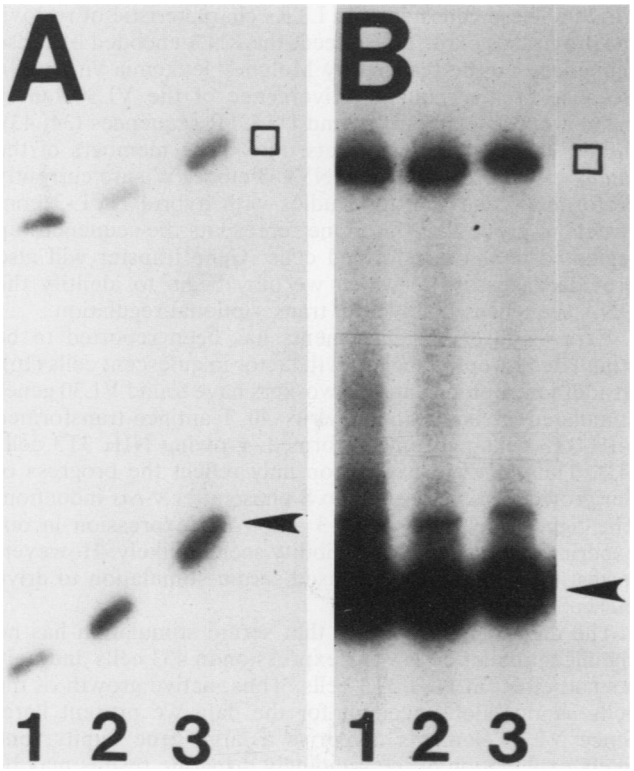

FIG. 5. Effect of serum stimulation on VL30 RNA accumulation in NIH 3T3 and 433 cells. Both panels contain S1 analyses of NVL-3 transcripts; the same probe shown in Fig. 4 was used. See text for details of serum stimulation. (A) RNA from NIH 3T3 cells stimulated with serum for 0, 2, or 4 h (lane 1, 2, or 3); (B) RNA from 433 cells stimulated with serum for 0, 2, or 4 h (lane 1, 2, or 3). Exposure of autoradiograms was for 14 h.

same treatment had no effect in 433 cells. These data also show that the basal level of NVL-3 RNA is 20-fold higher in 433 cells than in NIH 3T3 cells.

Run-on transcriptional analysis of genes in 433 cells. To determine whether the changes in steady-state RNA levels measured in the assays presented above have a transcriptional component, nuclei isolated from 433 cells have been used in run-on transcription assays (21). Results typical of these experiments are presented in Fig. 6. Quantitation of these experiments was accomplished by liquid scintillation counting of the [^{32}P]RNA which hybridized to the DNA probes immobilized on a nitrocellulose membrane; a tritiated M13 probe was included in the hybridization mix to serve as a control for the amount of hybridization (see Materials and Methods).

The run-on transcription analysis showed the following. (i) As expected, the rate of transcription of the MMTV-linked v-*ras* gene increases 20-fold 2 h after hormone treatment of 433 cells (Fig. 6A). This is not the case for NIH 3T3 cells (not shown). (ii) As predicted from the steady-state RNA analysis (see above), the levels of c-*myc* and c-*fos* transcription remain unchanged (Fig. 6A). (iii) Transcription of the v-*sis*-related gene shows an apparent increase of 1.5-fold (Fig. 6A). However, when the hybridization of data are analyzed as described above, no increase in transcription of these sequences is observed. In five separate experiments, including the ones shown in Fig. 6A and B, no increase in the transcription of v-*sis*-related sequences was observed. These data indicate that the mechanism by which steady-state v-*sis*-related RNA is increased (Fig. 3), must be post-transcriptional in nature. (iv) Transcription of NVL-3 increases fourfold after 2 h of glucocorticoid stimulation of 433 cells (Fig. 6B). This increase in transcription reflects the increase measured in steady-state NVL-3 RNA concentrations (see above). Hormone treatment of NIH 3T3 cells has no effect on NVL-3 transcription (data not shown).

DISCUSSION

We have utilized the glucocorticoid-responsive element of the MMTV LTR to promote the conditional expression of the Ha-v-*ras* gene. Thus, hormone stimulation causes 17-fold increases in v-*ras* mRNA and protein levels (Fig. 1 and 2) and results in loss of contact inhibition of growth and acquisition of the ability to grow in semisolid media (23). One problem with this system is that the glucocorticoid-regulated promoter does not maintain maximal transcription levels indefinitely. Instead, maximum transcription is reached after 2 to 4 h of hormone treatment, is maintained for an additional 6 to 8 h, and decreases fivefold after 12 to 16 h of hormone treatment (Fig. 1B). We have confirmed this result by using the run-on transcription assay and have also found that this effect is independent of the marker gene linked to the MMTV LTR (R. Owen and M. Ostrowski, unpublished observations). Other glucocorticoid-regulated genes, such as the mouse metallothionein-I gene, show a similar down regulation of steroid responsiveness after prolonged hormonal stimulation (24). It is possible that this down regulation may demonstrate a cellular control mechanism that limits the duration of hormone stimulation. However, since the effect of lowering v-*ras* expression is an unknown variable in our experiments, the effective use of

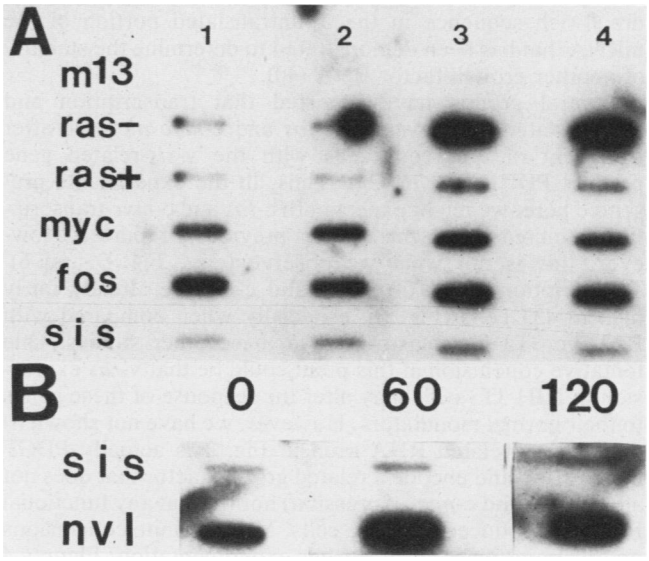

FIG. 6. Run-on transcription analysis of 433 cells. 433 cells were incubated with 10^{-7} M dexamethasone for the time indicated, in minutes, at the top of the samples. After run-on transcription (see Materials and Methods), labeled RNA was purified and hybridized to nitrocellulose filters that had cloned DNA samples immobilized to them. The identity of the DNA samples is given at the left. *ras*$^-$ and *ras*$^+$ correspond to the antisense and sense strands of the v-*ras* gene, respectively. Panels A and B represent two separate experiments. Lanes 1 to 4 in (A) represent 0, 1, 2, or 4 h of hormone treatment. The numbers at the top of the lanes in (B) represent the length of hormone treatment, in minutes. Autoradiography was for 12 h.

MMTV-driven *ras* expression is limited to a period of time 4 to 8 h after the initial hormone induction.

The conditional expression of v-*ras* in 3T3 cells allows the effects of this gene on cellular metabolic processes to be studied in a temporal fashion. The relevance of this point to studies concerning the action of oncogenes is apparent if transformation is perceived as a multistep process rather than a single event (4). In this view, the time window in which *ras* action is monitored is important because the cellular milieu in which this gene operates is progressively altered, ending in a fully transformed phenotype that is quite different from the progenitor cell.

We have chosen to use this system to study mouse genes whose expression is altered as intracellular *ras* concentrations increase. As detailed above, the literature contains examples of correlations between *ras* transformation and growth factor expression (1, 6). Since these growth factors are also able to affect gene expression (2, 26), molecular dissection of the primary effects of *ras* on gene expression from secondary effects is difficult without the ability to control *ras* expression.

The experiments presented here indicate that a selective and rapid alteration of gene expression can be correlated with conditional expression of the Ha-v-*ras* gene. Further, the regulation of this gene expression occurs at two levels: for v-*sis*-related sequences, regulation is post-transcriptional, while in the case of NVL-3 genes transcriptional regulation occurs.

The regulation of v-*sis*-related RNA concentrations at a post-transcriptional level adds another example to the growing list of genes modulated in this way (5, 18, 30, 41, 46). Although the exact nature of the regulation of v-*sis*-related gene expression awaits further experimentation, it is worth noting that the human c-*sis* gene contains an adenine-plus-uracil-rich sequence in the 3′-untranslated portion of the mRNA that has been demonstrated to determine the stability of another growth factor RNA (40).

Several groups have reported that transcription and steady-state RNA levels of c-*fos* and c-*myc* increase after treatment of quiescent cells with the v-*sis*-related gene product PDGF (19, 26, 29). Thus, in the experiments presented here, we might expect both c-*fos* and c-*myc* transcription to increase with the kinetics previously reported. However, this is not what we observe (e.g., Fig. 3 and 6). Transcription levels of c-*myc* and c-*fos* are already fairly high in 433 cells (Fig. 6), especially when compared with BALB/c 3T3 fibroblasts used in these other studies. One tentative conclusion at this point could be that v-*ras* expression in NIH 3T3 cells may alter the response of these genes to their normal modulators. However, we have not shown (i) that the *sis*-related RNA seen in Fig. 3 is actually PDGF mRNA (it could encode a related growth factor that does not affect c-*fos* and c-*myc* expression) nor (ii) that any functional PDGF is produced by these cells. More definite conclusions on this point must await further experimentation. Figures 4 and 6 contain data demonstrating that the NVL-3 gene is transcriptionally activated in 433 cells after stimulation of v-*ras* expression. Comparison of 1505 and 433 cells further strengthens the correlation between the expression of v-*ras* and NVL-3: a 17-fold difference in stimulated v-*ras* levels in these cells is reflected by a 20-fold difference in NVL-3 levels (Fig. 4, lane 2 versus lane 4 and lane 6 versus lane 8, respectively).

NLV-3 is a member of the VL30 mouse gene family (7). There are about 150 copies of VL30 elements in mouse cells; these elements exhibit considerable sequence divergence (9, 27, 28). These genes contain LTRs characteristic of retrovirus proviruses (7, 8, 27). Indeed, the RNA encoded by these sequences can be rescued by Moloney leukemia virus (39). Because of the sequence divergence of the VL30 family members, especially in U3 and U5 LTR sequences (34, 43), the S1 assay probably detects only those members of the family closely related to the NVL-3 clone. We are currently performing gene transfer studies with hybrid NVL-3 constructs to prove that this clone represents the sequences up regulated in *ras*-transformed cells. Gene transfer will also provide an assay by which we may begin to identify the DNA sequences involved in transcriptional regulation.

Expression of VL30 elements has been reported to be stimulated by epidermal growth factor in quiescent cells (16). In addition, Botchan and co-workers have found VL30 genes stimulated in both simian virus 40 T-antigen-transformed NIH 3T3 cells and untransformed, growing NIH 3T3 cells (43). Thus, NVL-3 expression may reflect the progress of our growth-arrested cells into S phase after v-*ras* induction. The tight coupling of NVL-3 and v-*ras* expression in our experiments makes this possibility seem unlikely. However, to test this hypothesis, we used serum stimulation to drive our cells into S phase.

The data in Fig. 5 shows that serum stimulation has no significant effect on NVL-3 expression in 433 cells and only a small effect in NIH 3T3 cells. Thus, active growth of the cells cannot alone account for the data we present here. Since VL30 elements comprise a large gene family, one likely explanation of the seemingly disparate results may be that different gene family members respond to different cellular stimuli. Consistent with this notion is the fact that the VL30 genes transcribed in simian virus 40 T-antigen-transformed cells have LTRs with sequences quite different from those of NVL-3 genes (34, 43). In fact, these sequences are most divergent in the regions assumed to contain the DNA signals involved in transcription initiation. Gene transfer experiments with chimeric constructions, as mentioned above, should help to resolve this issue.

The mechanisms by which *ras* p21 communicates with cellular regulatory networks is a topic important to understanding both cellular transformation and normal means of cell differentiation and maintenance. These mechanisms remain elusive at present. Whether changes in the expression of cellular genes play any role in these mechanisms is an intriguing but unanswered question. Our results on v-*sis*-related expression suggest that the induction of autocrine factors by v-*ras* might be involved in the transformation of cells by this gene. In addition, our work suggests a molecular genetic strategy to identify nucleic acid sequences and cellular factors involved in gene activation in *ras*-transformed cells.

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