ras Oncogene Activation of a VL30 Transcriptional Element Is Linked to Transformation

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The activity of a murine VL30 transcriptional element was increased 20-fold in transient assays by coexpression of mutant *ras* genes. The *cis* element did not respond to *ras* in a revertant cell line that was transformation defective. Therefore, *ras*-dependent alterations in transcription and *ras* transformation are linked. Deletion analysis of the VL30 long-terminal-repeat U3 region showed that a minimal 53-base-pair segment is required in *cis* for oncogene activation of transcription. Gel retention assays using a probe that contained the minimal *cis* element revealed that a unique complex was formed with nuclear proteins prepared from transformed cells. Exonuclease III footprinting and gel retention experiments that used oligonucleotide probes and competitors indicated that two distinct nuclear factors interact with the minimal *cis*-responsive element. Site-directed deletion of the 5'-proximal binding site (TGACTCT) resulted in a complete loss of *ras* responsiveness. However, deletion of this site did not affect stimulation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). These data are consistent with the hypothesis that *ras* and TPA signal transduction mechanisms for transcriptional activation are distinct.

Oncogene and proto-oncogene protein products can directly alter the expression of cellular genes. The evidence is especially compelling for oncogene products that are located in the cell nucleus. For example, the products of the *c-fos* and *c-jun* genes can collaborate to selectively alter the expression of cellular genes (9, 28). It is likely that such changes in gene expression are crucial for cell transformation directed by nuclear oncogenes (5).

Oncogene products that are not nuclear proteins can also affect the expression of genes. For example, *v-src* and *v-mos* have been reported to affect the expression of a mitogenic factor and collagen genes, respectively (34, 38). In addition, our laboratory has demonstrated that the Ha-*v-ras* gene product increases transcription of the NVL-3 mouse gene, a member of the VL30 gene family (27). Since the *v-ras* protein is found at the inner surface of the plasma membrane (3), these data imply a signaling pathway that connects the *ras* product to the cell nucleus. Defining this signaling pathway could provide important insights into how *ras* transforms cells.

Studies using the polyomavirus fibroblast enhancer element have shown that transient expression of a mutant *ras* allele or treatment of cells with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) can stimulate the activity of this element. This result has been interpreted to indicate that *ras* action is linked to the TPA-stimulated protein kinase C signal transduction pathway (39). Furthermore, a consensus API-binding site appears to mediate the effects of both agents (16), suggesting that *ras*, as previously demonstrated for TPA (2, 20), stimulates the activities of *fos* and *jun* oncogene products.

A nuclear target of *ras* that we used for this study is the NVL-3 gene. NVL-3 is a member of a family of mouse genes that are termed VL30 elements (7). These elements resemble the integrated proviral form of retroviruses (7, 11, 17). They are present at about 150 to 200 copies per genome, and the different members exhibit sequence heterogeneity, particu-

larly in the long-terminal repeat (LTR) sequences (26, 36). VL30 genes can respond to various cell growth signals. For example, a single VL30 element can respond independently to two different growth signals, TPA and epidermal growth factor (30).

In this work, we present evidence demonstrating that a small region of the NVL-3 LTR is a nuclear endpoint for *ras* action. We show that activation of the NVL-3 element and, by extension, a signaling pathway that connects *ras* to the nucleus is linked to the ability of *ras* to transform cells in culture. In addition, we present evidence implying that different nuclear factors and different signal transduction pathways mediate *ras* and TPA responses.

MATERIALS AND METHODS

Cell lines and cell culture. NIH 3T3, DT, and C11 (25) cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 9.5 mM L-glutamine, and 100 µg of mezlocillin per ml. The DT and C11 cell lines are derivatives of NIH 3T3 cells that express Ki-*v-ras* (25). In some experiments, cells were treated with TPA at a concentration of 200 ng/ml with 0.1% (vol/vol) dimethyl sulfoxide (DMSO). Cells not treated with TPA were treated with 0.1% (vol/vol) DMSO. RNA from these cell lines was isolated and analyzed as described previously (27).

Recombinant DNA constructions. Standard methods were used to subclone DNA sequences (22). The construct pRDO19 was made by inserting the U3 region of NVL-3 (−17 to −264 with respect to the RNA cap site [26]) and the upstream basal promoter element of the mouse major β-globin gene (−335 to +1 with respect to the RNA cap site [8]) into the *Bgl*II site of the firefly luciferase plasmid pJD205 (13). The transcriptional orientation of these elements is shown in Fig. 1. Plasmid pRDO25 was constructed by deleting the NVL-3 sequences from pRDO19 and replacing it with a polylinker that contains *Xho*I, *Pst*I, and *Hind*III restriction sites. These sites were used for construction of polymerase chain reaction (PCR) deletion mutants (see

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FIG. 1. Plasmid DNA constructs used for transfection experiments. Plasmids pRDO19 (A), pRDO25 (B), pDB2 (C), and pDB3 (D) were constructed by using pJD205, which contains the firefly luciferase gene (13). pRDO25 was constructed by adding to pJD205 a 300-bp *Hind*III-to-*Bgl*II restriction fragment containing the mouse major β -globin promoter (8) immediately 5' to the luciferase gene. pRDO19 differs from pRDO25 in the presence of a 242-bp *Bam*HI-to-*Hind*III restriction fragment containing the U3 region of the NVL-3 LTR (7), located 5' to the β -globin promoter. pDB2 and pDB3 were derived from pRDO19 and pRDO25, respectively, by inserting the RSV enhancer (21) 3' to the luciferase gene.

below). pDB2 and pDB3 were constructed by inserting a *Pvu*II-*Eco*RI restriction fragment that contains the 3' LTR of Rous sarcoma virus (RSV), including the enhancer element (21), into the unique *Bam*HI sites 3' of the luciferase gene in pRDO19 and pRDO25, respectively. This enhancer element increased the basal level of expression of the luciferase expression vectors in DT, 3T3, and C11 cells. Since C11 cells were difficult to transfect (see below), pDB2 and pDB3 were useful in experiments that included these cells.

The PCR (Perkin Elmer Cetus) was used to generate the deletion constructs shown in Fig. 4 and 7. Oligonucleotides that contained NVL-3 U3 sequences were synthesized by using an Applied Biosystems 380A DNA synthesizer and then purified by high-pressure liquid chromatography. The oligonucleotides used to define the 5' boundaries of the constructs used were (5' to 3') CTCGAGGAAGAATAAAA AATTACTGG (pRDO48 and -49), CTGCAGCCACAGAAT GTTTC AACAGG (pRDO39), CTGCAGGTTGTTAAACTT CCCCTATT (pRDO45), and CTGCAGGTTGTTAACTTC CCCTAT (pRDO46). Oligonucleotides used to define 3' ends of deletions were AAGCTTTTAAAGGAAAAAAC (pRDO39, -45, and -46), CTGCAGAGTCATATCCTGTGTT TG (pRDO50), CTGCAGGAATAGGGGAAGTTTAACAA (pRDO49), and CTGCAGCCTGTGTTTGGCAATACATC (pRDO48). The PCR products were cut with *Pst*I and *Hind*III for 5' deletions and with *Xho*I and *Pst*I for 3' deletions and cloned into the pRDO25 polylinker (see above). The deletion endpoints were thus defined by *Pst*I sites. Plasmids pRDO51 and pRDO53 were made by joining pRDO45 with pRDO48 and pRDO46 with pRDO50, respectively, at the unique *Pst*I sites. These internal deletions are therefore similar to linker-scanning mutations; however, such deletions had more NVL-3 information deleted than

was replaced by the *Pst*I site (see Fig. 8). The entire NVL-3 region of all PCR-generated deletions and linker-scanning mutations were sequenced by using the Sequenase kit (U.S. Biochemicals Corp.).

Transfection of cells with plasmid DNA. Transfections were performed by methods previously described (18), with the following modifications. Cells were plated on collagen-coated dishes at 3×10^5 to 5×10^5 cells per dish. A total of 10 μ g of DNA was added to each dish. As an internal control, a plasmid containing a secreted form of the human placental alkaline phosphatase (SEAP) gene driven by the RSV LTR (4) was included in the DNA precipitates. SEAP activity was not affected by *ras* expression (data not shown). The amount of SEAP included in the experiments was chosen so that competition effects were not observed.

For transient expression of *ras*, the expression vector pHO6T1 (37), containing the human Ha-c-*ras* EJ bladder carcinoma gene, was used. As the control for this vector, the expression vector with *ras* sequences deleted (Homer 6 [37]) was included. In experiments comparing 3T3, C11, and DT cells, 9 μ g of reporter and 1 μ g of SEAP plasmid were used for each dish. In cotransfection experiments, each plate received 4.5 μ g of reporter gene, 4.5 μ g of expression vector, and 1 μ g of SEAP plasmid. CaPO_4 -DNA coprecipitates were removed after 12 to 20 h by washing with Tris-buffered saline (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl) containing 3 mM ethylene glycol-bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA). Fresh medium supplemented with 10% FBS was then added to the cells. Cells were harvested 36 to 48 h later. In the *ras* cotransfection and TPA experiments presented in Fig. 8, medium containing 10% FBS was replaced with medium containing 0.5% FBS 16 h before harvest.

Enzyme assays used for analysis of transfection experiments. Luciferase was extracted from cells by lysis with the detergent Nonidet P-40. Lysis buffer contained 100 mM KH_2PO_4 (pH 7.8), 3 mM MgCl_2 , 1% (vol/vol) Nonidet P-40, and 1 mM dithiothreitol. Insoluble matter was removed from the lysate by centrifugation, and protein concentration of extracts was determined with the Bradford assay (Bio-Rad Laboratories). Luciferase activity was determined as follows: samples of extracts were adjusted to 30 mM glycylglycine (pH 7.8)–10 mM MgSO_4 –6 mM ATP–1.3 mg of protein per ml (by adding bovine serum albumin) in a volume of 300 μ l. A Biolumat LB 9500 C luminometer (Berthold Analytical Instruments) was used to measure peak luminescence upon addition of luciferin to 625 μ M. Luciferase data were normalized for protein concentration and for relative transfection efficiency as determined by SEAP assays (see below). For each extract, luciferase assays were performed multiple times, using different amounts of extract to ensure that the data were gathered in the linear range of the assay.

SEAP activity was measured essentially as described previously (4), with the following modifications. Medium samples were heat treated for 10 min and clarified by centrifugation for 5 min. A_{405} was measured by using a Bio-Rad 2550 EIA reader.

Normalized luciferase activity was expressed as luminescence per microgram of protein extract divided by milliunits of SEAP activity per milliliter of culture medium. The range of transfection values presented varied by less than 10%.

Analysis of nuclear factors. Nuclear extracts were prepared as described previously (18) except that isolated nuclei were extracted with a 400 mM salt wash. DNA gel retention assays were performed as described previously (18), with some modifications. Binding reactions had a volume of 20 μ l

and contained 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 50 mM NaCl, 12% glycerol, 1 mM dithiothreitol, and 4 μ g of poly(dI-dC). Extract was preincubated with the reaction components listed above for 5 min at room temperature and incubated for another 5 min at room temperature upon addition of probe. The amount of extract used is indicated in the figure legends.

Exonuclease III footprinting reactions were carried out as described previously (18) with enzyme supplied by Boehringer Mannheim Biochemicals. Oligonucleotides used as competitors for exonuclease III and gel retention experiments were ATGACTCTTTG and CAAAGAGTCAT or TG GTTGAGTAAATTTGTGG and CCACAAATTTACTCAA CCA. Complementary oligonucleotides were adjusted to 100 mM NaCl at a concentration of 100 μ g/ml, heated to 95°C for 1 min, and slowly cooled to room temperature to ensure that they were double stranded when used.

RESULTS

Activity of NVL-3 U3 sequences in *v-ras*-transformed cells. In previous work, we showed that expression of the *ras* oncogene product leads to increased transcription of the mouse NVL-3 gene (27). We next wished to identify the *cis*-acting DNA elements that mediate NVL-3 *ras* responsiveness. To accomplish this, the ability of the U3 region of the NVL-3 LTR to confer *ras* responsiveness to a heterologous promoter element was studied.

For these experiments, the chimeric reporter plasmids shown in Fig. 1 were constructed. These plasmids contain a β -globin promoter (8) linked to the firefly luciferase gene (13). pRDO19 and pDB2 contain the NVL-3 U3. Two of the constructs (pDB2 and pDB3) contain the RSV enhancer (21). This enhancer is not *ras* responsive (data not shown). The RSV enhancer was included to increase the basal-level activity of the luciferase constructions. This was especially useful in the transient assays in C11 cells, since these cells were transfected with much lower efficiency than were either DT or NIH 3T3 cells.

Recombinant reporter plasmids were transfected into NIH 3T3 cells, Ki-*v-ras*-transformed DT cells, and C11 cells. C11 is a mutant cell line that expresses *v-ras* but is revertant for the transformed phenotype (25). In *ras*-transformed DT cells, the NVL-3 U3 sequence enhanced the expression of luciferase activity 10-fold relative to the construct that lacked these sequences (Fig. 2). In the nontransformed NIH 3T3 and C11 cells, the U3 sequence enhanced heterologous gene expression no more than twofold on average. All transfection experiments included SEAP internal controls (see Materials and Methods). These results demonstrate that the U3 portion of the NVL-3 gene is capable of conferring a *ras*-enhanced increase in expression of a heterologous gene and that the *ras*-induced increase in U3 activity is linked to transformation.

The activity of the endogenous NVL-3 gene resident in each of the three cell lines was tested by S1 mapping (Fig. 3B) to confirm that the U3 activity measured in the transient assay was reflected by the activity of the endogenous gene. Published sequence data indicate that the NVL-3 U3 and R regions are quite different from those of other members of the VL30 gene family (26, 36). Therefore, stringent S1 analysis should detect only the NVL-3 gene. Results obtained with the chimeric plasmids did reflect the activity of the endogenous gene: NVL-3 expression was found in NIH 3T3 and C11 cells but was approximately 20-fold higher in transformed DT cells (Fig. 3A, lanes 1 and 5 versus lane 3).

FIG. 2. Activity of the NVL-3 U3 region in cell lines that stably express *v-ras*. Plasmids pDB2 and pDB3 were transiently transfected into NIH 3T3, DT, and C11 cells. Protein extracts made from the cells were analyzed for luciferase activity. Data are averages from four experiments. Activity is expressed on the y axis as the ratio of luciferase activity in cells transfected with pDB2 to that in cells transfected with pDB3 for each cell line.

In parallel, NIH 3T3, DT, and C11 cells were grown in the presence of the tumor promoter TPA, and the effect on NVL-3 expression was measured by S1 assay (Fig. 3A, lanes 2, 4, and 6). NVL-3 expression was increased by TPA in 3T3 and C11 cells but not in DT cells. TPA stimulation in 3T3 and C11 cells was rapid and peaked within 2 h after drug administration (data not shown). Thus, the NVL-3 gene was not *ras* responsive in C11 cells but was responsive to TPA action.

U3 *cis*-regulatory function is confined to a 53-base-pair (bp) sequence. Progressive 5' and 3' deletions of NVL-3 U3 sequences were constructed to further delineate the sequence requirements for *ras*-mediated transcriptional regulation. The mutants were made by the PCR (see Materials and Methods) so that a small set of deletions with defined ends could be generated and tested.

The deletions were cloned into the pRDO25 (no RSV enhancer) background (Fig. 1), and their activities were tested by transient transfection assays (Fig. 4). An expression vector that contained a mutant human Ha-*ras* oncogene (37) was cotransfected with the chimeric luciferase constructs, and the empty expression vector was cotransfected in parallel. Each transfection was performed independently at least four times, and SEAP plasmid was included in each cotransfection as an internal control.

Data from transient transfections performed with pRDO19 showed that the ability of the U3 to enhance expression of the luciferase gene was 20-fold higher in the presence than in the absence of *ras* expression. Therefore, the U3 element responded to both transient and stable expression of *ras* (see above).

Deletion of 127 bp from the 5' end of the U3 (pRDO39) preserved the sequences that responded to *ras* expression, whereas deletion of 161 bp from the 5' end of the U3 (pRDO45) abolished any induction by cotransfected *ras*. Deletion of 67 bp from the 3' end of the U3 (pRDO49) preserved induction, but deletion of 98 bp from the 3' end of the U3 (pRDO48) left no appreciable *ras* inducibility. Together, the results of these experiments define the minimal

FIG. 3. Effect of TPA treatment on NVL-3 activity. NIH 3T3, DT, and C11 cells were treated with TPA (200 ng/ml) dissolved in DMSO or with DMSO alone for 2 h. RNA was isolated from cells, and 10 μ g was hybridized with the NVL-3 probe diagramed in panel B. This probe was a 483-bp restriction fragment labeled at an *Xho*I site (*). Correctly initiated transcripts that were hybridized to the probe and digested with nuclease S1 should yield a 221-bp radiolabeled fragment. (A) Autoradiogram of a denaturing 5% polyacrylamide gel used to analyze S1-resistant radiolabeled fragments. RNA samples: NIH 3T3 (lane 1), TPA-treated NIH 3T3 (lane 2), DT (lane 3), TPA-treated DT (lane 4), C11 (lane 5), and TPA-treated C11 (lane 6). $\rightarrow$, Mobility of the major protected band. Lane M, *Msp*I-digested pBR322 DNA size markers.

ras-inducible *cis*-regulatory sequence to the 53 bp from -84 to -137 with respect to the site of NVL-3 transcription initiation. Transfection of the deletions into 3T3 versus DT cells established the same minimal boundaries for the *ras*-responsive element (data not shown).

In the absence of a cotransfected *ras* gene, the basal-level activities of pRDO19, pRDO39, and pRDO49 were comparable. The basal-level activities of pRDO45 and pRDO48 were about 20% of the pRDO19 basal activity. Thus, the 53-bp *cis*-acting element that mediated *ras* stimulation was also important for basal-level transcriptional activity.

Analysis of nuclear factors that bind to the U3 *cis*-regulatory sequence. Nuclear extracts made from both normal and transformed cells were screened for the presence of factors that bound specifically to U3 DNA in gel retention assays (14). The probe for this analysis was a 115-bp *Hae*III-*Hind*III restriction fragment corresponding to the U3 sequences from bp -137 to -22 and therefore contained all sequences required for *ras* action as defined in the deletion analysis presented above. One complex, termed vl 2, was unique to DT nuclear extracts (Fig. 5, lane 1). A distinct complex, labeled vl 1, was found in NIH 3T3 extracts (lane 2).

Competition experiments with unlabeled U3 DNA probe (Fig. 5, lanes 3 and 4), nonspecific plasmid DNA (lanes 5 and 6), or a 50-mer containing the glucocorticoid response element of mouse mammary tumor virus (lanes 7 and 8; coordinates -190 to -140 with respect to the mammary tumor virus cap site [18]) were performed. Complexes vl 1 (NIH 3T3 extract) and vl 2 (DT extract) could be specifically abolished by excess U3 DNA but not by the other two competitors. Other complexes visible in lanes 1 and 2 resulted from nonspecific protein-DNA interactions, since both specific and nonspecific competitors could inhibit their formation. These results demonstrated that complexes vl 1 and vl 2 resulted from specific interactions between nuclear factors and U3 DNA.

TPA treatment of C11 cells resulted in increased expression of the endogenous NVL-3 gene (Fig. 3). To examine whether the same nuclear factors were responsible for this TPA induction as were responsible for the *ras*-mediated induction found in DT cells, nuclear extracts were prepared from untreated and TPA-treated C11 cells and used in gel retention assays. The pattern of bands observed with either C11 extract was identical to the pattern obtained with NIH

FIG. 4. Deletion analysis of the NVL-3 U3 region. Deletion mutants were generated by using the PCR. Boundaries of the various deletions are indicated by arrowheads and numbers. The deletions were placed in pRDO25 (see Materials and Methods). NIH 3T3 cells were transiently transfected with each plasmid. Cotransfected with the luciferase plasmids were either the Val-12 Ha-c-*ras* expression plasmid or the expression plasmid with oncogene sequences deleted. Protein extracts derived from transfected cells were analyzed for luciferase activity. The relative luciferase activity of each cotransfection is shown by horizontal bars as percentage of the luciferase activity in extracts made from cells cotransfected with pRDO19 and the Ha-c-*ras* expression plasmid.

FIG. 5. Detection of specific NVL-3 U3-binding activity in nuclear protein extracts. (A) Nuclear extracts from NIH 3T3 and DT cells (5 μ g of protein) were incubated with a 115-bp radiolabeled DNA probe corresponding to the NVL-3 U3 sequence from bp -137 to -22. Protein-DNA complexes were resolved by gel electrophoresis and visualized by autoradiography. The source of nuclear extract (NIH 3T3 or DT cells) and the identity of competitor DNA are indicated above each lane. Lanes U3 contained a 20-fold molar excess of unlabeled probe DNA; lanes pUC contained a 20-fold molar excess of restriction enzyme-digested pUC plasmid DNA; lanes GRE contained a 20-fold molar excess of a double-stranded oligonucleotide containing the glucocorticoid response element (18). vl 1 and vl 2 are the major complexes specific to NIH 3T3 and DT extracts, respectively. (B) NIH 3T3, C11, and DT cells were treated with DMSO; C11 cells were treated with DMSO and 200 ng of TPA per ml. Nuclear extracts from these cells were used in binding reactions with a 115-bp probe as described above. The source of extract and the presence (+) or absence (-) of TPA treatment are indicated above each lane. Complexes vl 1 and vl 2 are labeled.

3T3 extract (Fig. 5B, lanes 1 to 3). This pattern was clearly distinct from that found with DT cell extracts (lane 4). TPA treatment had no effect on the presence or quantity of vl 1 or vl 2 in NIH 3T3 or DT cells, respectively (data not shown).

Exonuclease III footprinting was used to identify the sequences, at the nucleotide level, that were specifically recognized by nuclear factors present in DT cells. Various amounts of DT extract were combined with the 115-bp U3 DNA probe in binding reactions and then subjected to partial exonuclease III digestion. The minus DNA strand (bottom strand in Fig. 6C) was 5' end labeled for this analysis. Figure 6A shows the results of exonuclease III footprinting of U3 sequences. With increasing amounts of extract, three major boundaries of protection were observed (arrows in Fig. 6A). Of the regions of the NVL-3 sequence that were protected in this footprinting experiment (arrows in Fig. 6C), two of the three major areas lay within the minimal *cis*-regulatory sequence defined in the transfection analysis of deletion mutants described above. These data also suggest that the factor recognizing the 5' distal region was present in lower concentrations in the extracts than the factors recognizing the two downstream sequences, since higher protein concentrations were required to observe exonuclease III footprints of comparable intensity. Alternatively, the protein binding to the 5' distal region may have lower affinity for the U3 DNA.

An exonuclease III footprinting experiment was performed with oligonucleotide competitors to demonstrate specificity (Fig. 6B). The sequences of the competitors are indicated by bars in Fig. 6C. Each oligonucleotide specifically inhibited the formation of only one of the footprints.

Oligonucleotides as probes for factors present in transformed cells. To address the implications of the exonuclease III data described above, two oligonucleotides were synthesized: CCAGATGTATTGCCAAACACAGGATATGACTC TTTGGT and ACCAAAGAGTCATATCCTGTGTTGGC AATACATCTGG. These complementary oligonucleotides contain the upstream binding site described by exonuclease III digestion. The sense-strand oligonucleotide was 5' end labeled and hybridized to the complementary strand as described in Materials and Methods. This double-stranded DNA was used in gel retention assays with extracts produced from either DT or C11 cells grown in the presence of TPA. DT and C11 extracts contained distinct factors that recognized this DNA probe (Fig. 7). Significantly, the major complex seen in DT cells migrated with electrophoretic mobility different from that of the major complexes observed in C11 cells. However, at least two complexes of the same mobility were seen in both extracts (Fig. 7). Competition with a 20- or 40-fold molar excess of cold, homologous oligonucleotide resulted in decreased binding by the major and several minor binding activities seen in these extracts, including the major complex from DT cell extracts. An oligonucleotide representing the downstream binding site (B in Fig. 6C; see Materials and Methods for the precise sequence) did not compete the formation of major complexes derived from either cell line. The findings represented in Fig. 7 have been repeated with different preparations of probe and competitor and with extracts made over an 18-month period.

Deletion of specific sequences that confer *ras* inducibility upon the U3 sequence. To test the biological activity of sequences that were implicated by gel retention and footprinting assays, we constructed reporter plasmids containing mutations similar to linker-scanning mutations (see Materials and Methods). Figure 8C shows the U3 sequence in the vicinity of the mutations as well as the specific sequences present in two of the test plasmids, pRDO51 and pRDO53.

NIH 3T3 cells were transiently transfected with reporter plasmids containing no U3 sequence, wild-type U3 sequence, or one of the mutations. Each reporter was cotransfected with either the *ras* expression plasmid or a control plasmid (see above). Each cotransfection included a SEAP internal control. Cells cotransfected with pRDO19, which contains the wild-type U3 sequences, and the *ras* expression plasmid contained 17 times as much luciferase as did cells cotransfected with pRDO19 and the control plasmid (Fig. 8A). The luciferase activity provided by the mutant pRDO53 was 10-fold higher in the presence than in the absence of cotransfected *ras*. The *ras* inducibility of mutant pRDO51 was not appreciably greater than that of control plasmid pRDO25, which contains no U3 sequences. pRDO19 and pRDO53 had similar basal levels of activity, but the basal-level activity of pRDO51 was 25% of the activity of pRDO19. The phenotypes of the two mutant NVL-3 elements were the same in DT cells as in the cells that transiently expressed *ras* (data not shown).

We next tested the TPA inducibility of pRDO19, pRDO25, pRDO51, and pRDO53. NIH 3T3 cells were transiently transfected with each plasmid in the presence and absence of TPA (Fig. 8B). The values shown were normalized to those for SEAP internal controls. Luciferase activity from

FIG. 6. Exonuclease III footprinting of sequence-specific nuclear factors. Nuclear extract from DT cells was used to perform binding reactions as described for the gel retention assay. The probe used (see legend to Fig. 5) was labeled on the bottom (minus) strand only. Probe complexes were subjected to partial digestion with exonuclease III. The digestion products were resolved by electrophoresis through denaturing 8% polyacrylamide gels. After drying, gels were autoradiographed. In panel A, the amount of extract (in micrograms) used in binding reactions is indicated above the appropriate lane. Lane M, *Msp*I-digested pBR322 DNA size markers, the sizes of which are indicated on the left in bases. Arrows mark the boundaries of exonuclease III-protected regions. (B) Autoradiogram from a competition experiment. A 5- μ g sample of DT extract was used for each reaction. Double-stranded oligonucleotide competitors were included in the binding reactions for the samples in lanes A and B. (C) Diagram of the U3 sequence from bp -139 to -55 with respect to the transcriptional start site. Sequences with similarity to consensus AP1-binding sites are boxed; the three major boundaries of protection from exonuclease III digestion shown in panel A are indicated. Sequences of the competitor DNAs referred to in panel B are indicated by bars A and B.

pRDO19 was induced approximately fivefold by treatment of cells with TPA. The mutations present in plasmids pRDO51 and pRDO53 had no significant effect on the fivefold induction of luciferase activity caused by TPA treatment. As indicated above, the basal level of pRDO51 was fourfold lower than that of pRDO19 or pRDO53. Luciferase activity provided by transfection of pRDO25 was not induced by TPA treatment.

DISCUSSION

NVL-3 contains a transformation-dependent regulatory element. Expression of the NVL-3 gene selectively increases when the level of Ha-v-*ras* expression is increased in NIH 3T3 cells (27). This increase in NVL-3 expression is the result of an increase in the rate of NVL-3 transcription, the kinetics of which closely resemble those of Ha-v-*ras*. In addition, work from different laboratories has demonstrated that *ras* can effect the transcription of viral and cellular genes (35, 39). Since the *ras* gene product is located at the inner surface of the plasmid membrane (3), these results indicate the existence of a signal transduction pathway(s) that links oncogene action to the nucleus. Our goal in studying *ras* activation of nuclear events is to define this signal transduction pathway. To do this, we must first define the *cis*-acting DNA elements and *trans*-acting factors that constitute one endpoint of *ras* action.

In this work, we demonstrate that sequences contained within the NVL-3 U3 region respond to *ras* action in cells that are transformed and stably expressing the p21 gene product (Fig. 2 and 3). In revertant C11 cells, *ras* p21 is expressed, but the cells are no longer transformed (25). U3 activity correlates not to *ras* expression in C11 cells but to the transformed phenotype of cells (Fig. 2 and 3). Apparently, the dominant gene product that suppresses *ras* transformation in the C11 cell line (25) also suppresses the signal transduction pathway connecting *ras* to certain nuclear events.

The work of another group has demonstrated that transient coexpression of *ras* can increase the activity of the polyomavirus fibroblast enhancer (39). However, the polyomavirus enhancer is not activated by *ras* in stably transformed cell lines (33). Another group has shown that transcription of the *c-fos* gene increases during a narrow time frame after the onset of v-*ras* expression in cultured cells (35). The *c-fos* promoter and the collagenase enhancer were also shown to be *ras* responsive in transient coexpression assays (35).

Such experiments indicate that *ras* selectively activates viral and cellular transcription elements. However, the biological relevance of such activation, and thus of the signal transduction pathways involved, to *ras* transformation remains uncertain. In contrast to these results, data presented

FIG. 7. Analysis of nuclear factors in transformed cells, using an oligonucleotide probe. The oligonucleotide probe representing the upstream binding site (see text) was end labeled and used in gel retardation assays. Extracts were prepared from DT cells and from C11 cells grown in the presence of TPA; 5 μ g of extract and 0.2 ng (50,000 dpm) of probe was used per reaction. Lanes A had a 20- or 40-fold molar excess of unlabeled probe DNA included in the reaction mix; lanes B contained a 20- or 40-fold excess of an oligonucleotide representing the downstream binding site (B in Fig. 6C; see Materials and Methods). Large and small arrowheads represent major and minor complexes, respectively, observed only in DT extracts. □, Complexes observed only in C11 extracts; ■, complexes seen in both types of extracts.

here demonstrate that a pathway exists in stably transformed cells that selectively links *ras* p21 to the nucleus. Our results with C11 cells (Fig. 2 and 3) clearly link *ras* transformation of cells in culture and the signal transduction pathway between *ras* and the nucleus.

Nuclear factors present in transformed cells recognize the *ras*-responsive element. Deletion analysis of the NVL-3 U3 limits the target for *ras* action to a 53-nucleotide region of DNA (Fig. 4). This same region of DNA also contains the information required for the basal-level activity of the U3 enhancer (Fig. 4; compare pRDO39 and pRDO45). Gel retardation assays with nuclear extracts prepared from transformed DT cells indicate the presence of a single major specific complex between nuclear factors and the *ras* target sequences that we have called vl 2 (Fig. 5A). This complex does not form when nuclear extracts from nontransformed 3T3 or revertant C11 cells are used. Instead, a specific complex of lower electrophoretic mobility is observed (vl 1; Fig. 5B).

Footprinting experiments using exonuclease III indicate that at least three factors recognize sequences in the U3

DNA used as a probe for the gel retardation assays. Two of these are located within the minimal oncogene target sequences (Fig. 6A). Inspection of the protected regions reveals that both sites contain sequences that match 6 of 7 bp in the consensus binding site for transcription factor AP1, TGACTCT and TGAGTAA (Fig. 6B). AP1 can bind to sequences found in other enhancers that are identical to both of these sites (2, 16, 19, 20, 23).

Competition footprinting experiments demonstrate that different factors in DT cells must recognize the two binding sites (Fig. 6B). These results show that short oligonucleotide competitors can block formation of only the homologous exonuclease III footprint; e.g., probe A inhibits the formation of only the 5' distal exonuclease III boundary (Fig. 6B, lane A), whereas probe B blocks formation of only the downstream boundary (Fig. 6B, lane B).

The results of gel retention experiments with an oligonucleotide probe (Fig. 7), showing that distinct complexes are formed in DT versus C11 cell extracts, confirm the nuclease protection data. The major complex detected in DT cells is probably identical to the complex labeled vl 2 in Fig. 5. Formation of this complex is inhibited by the homologous oligonucleotide but not by one containing the downstream binding site. Thus, two distinct factors must recognize the related AP1-like sequences. The pattern of bands detected is actually quite complex (Fig. 7), which may reflect the fact that a family of nuclear factors are capable of recognizing AP1-like sequences (see below). Clearly, a more detailed biochemical analysis will be necessary to adequately address this issue.

The phenotypes of site-specific mutants provide additional evidence that the binding sites are distinct. Deletion of the 5' distal binding site in pRDO51 results in the loss of *ras* activation, whereas deletion of the downstream binding site in pRDO53 has no effect on oncogene modulation of NVL-3 activity (Fig. 8A). Mutation of the 5' distal site also decreases the basal level activity of the U3 element three- to fourfold. In contrast, the activities of both pRDO51 and pRDO53 are increased fivefold by TPA treatment (Fig. 8B).

***ras* signal transduction can be distinguished from TPA signal transduction.** Imler et al. (16) have shown that an AP1 consensus sequence is important for *ras*-modulated activation of the polyomavirus fibroblast enhancer. The polyomavirus AP1 element appears to be responsible for both oncogene and TPA regulation (16, 39). On the basis of this evidence, the authors have proposed that the *ras*-mediated pathway which leads to nuclear activation events is identical to the TPA-inducible pathway (presumably a protein kinase C-dependent pathway [24]). We present here three lines of evidence that are inconsistent with this hypothesis.

First is the behavior of the endogenous NVL-3 gene in the mutant C11 cells (Fig. 3). In this cell line, the response of the endogenous gene to *ras* expression is interrupted (Fig. 3; compare lanes 3 and 5). TPA treatment of C11 cells induces expression of the endogenous NVL-3 gene (lane 6). This interpretation depends on the ability of the S1 to distinguish NVL-3 from other members of the VL30 gene family present in mouse cells. As pointed out in Results, the S1 assay should be capable of doing this: members of the VL30 family sequenced thus far have distinct U5 regions (26, 36). However, even if members of the VL30 family other than NVL-3 are detected, these genes respond to TPA stimulation but not to *ras* in C11 cells (Fig. 3). Thus, the mutation in C11 cells separates the effects of *ras* and TPA on VL30 gene activity.

Second is the analysis of nuclear factors that form specific complexes with U3 sequences (Fig. 5). If TPA and *ras* signal

FIG. 8. Analysis of NVL-3 U3 site-specific internal deletion mutants. (A) *ras* induction. NIH 3T3 cells were transiently transfected with pRDO19, pRDO51, pRDO53, and pRDO25. Each plasmid was cotransfected with a SEAP internal control as well as either a Ha-c-*ras* expression plasmid (■) or an empty expression plasmid (□). Data are averages from four separate transfection experiments. The fold induction of luciferase activity produced by *ras* expression is indicated to the right of each pair of bars. (B) TPA induction. Each of the four plasmids listed above was cotransfected with a SEAP internal control plasmid as well as Homer 6 (37). Cells were treated with DMSO alone (□) or with DMSO and TPA (■). Data are averages from four separate transfection experiments. The fold induction of luciferase activity produced by TPA treatment is indicated to the right of each pair of bars. Cells were switched to medium containing 0.5% FBS 16 h before harvest for all experiments shown. (C) Sequence of the NVL-3 U3 from bp -119 to -80 (large letters). pRDO19 contains the wild-type U3 sequence. The sequences of pRDO51 and pRDO53 diverge from the wild-type sequence only in the region shown. Deletions are indicated by dashed lines; base replacements are indicated by small letters.

transduction are identical, then gel retention complexes formed in nuclear extracts prepared from TPA-treated C11 or DT cells should be the same. This is not what we found (Fig. 5B [compare lanes 3 and 4] and 7). In addition, the studies performed with the oligonucleotide probe (Fig. 7) indicate that the two binding sites can be distinguished *in vitro*. The data imply that different nuclear factors are activated by TPA and *ras* and that *ras*-activated factors can distinguish between AP1-like sequences.

Third is the phenotype of the site-specific deletion mutations present in pRDO51 and pRDO53 (Fig. 8). Deletion of the 5' distal site (TGACTCT) in pRDO51 results in the loss of responsiveness to oncogene action. However, pRDO51 remains TPA responsive. Thus, the sequence requirement for oncogene activation can be different from the TPA requirement.

One simple hypothesis to account for our results is that the *ras* and TPA responses are distinct. Since an AP1-related recognition sequence is involved, *ras* stimulation of transcription is likely to involve members of the *fos* and *jun* families.

The *c-jun* oncogene is a member of a multigene family encoding related proteins (31, 32). Likewise, *c-fos* belongs to a multigene family (10). *In vitro* biochemical evidence reveals that members of these families can form *jun* homo- or heterodimers and *fos-jun* heterodimers. These dimers can all bind to AP1 consensus sequences (15, 23, 28). *In vivo* evidence demonstrates that such interactions are also important for activation of enhancers containing AP1 sites (9). Such data suggest that a plethora of regulatory combinations may be defined by *fos-jun* interactions, all dependent on recognition of AP1 consensus sites.

AP1 sites can mediate TPA transcriptional responses (2, 20). Therefore, signal transduction pathways impinge on *fos*

and *jun* transcriptional activation. The manner in which various signal transduction pathways interact with *fos* and *jun* gene families may have important implications for many biological phenomena, including cellular differentiation and the control of cell growth (12). Viewed in this manner, it appears overly simplistic to equate action through an AP1 *cis* element with identity in signal transduction pathways. Careful biochemical and molecular genetic analysis will be required to determine which members of the *fos* and *jun* families interact with specific signal transduction pathways. The data we present are consistent with the idea that TPA and *ras* may activate distinct signal transduction pathways and thus distinct members of the *fos* and *jun* families. However, more careful analysis of the proteins present in the DT extracts will be needed to prove that this is the case. Such experiments are now under way in our laboratory.

Mutation of *c-ras* oncogenes is strongly correlated with the progression of many human neoplasias (3). For example, *ras* gene mutation is found in most endocrine pancreatic and in 40% of colon tumors (1, 6). Therefore, an understanding of what mutant and normal *ras* alleles do in cells seems paramount in developing therapeutic strategies for such human diseases. Our approach to characterizing mutant *ras* function is to define an endpoint of oncogene action. Conceptually, such an approach is identical to the classic strategy that led to the identification of cyclic AMP as a second messenger of epinephrine action (reviewed in reference 29). The evidence presented in this report defines one endpoint of *ras* action and thus provides a framework in which to study a particular aspect of oncogene function at the molecular level. In conclusion, we suggest that the *ras* nucleus signaling pathway may be a target for developing chemotherapeutic agents specific for preventing *ras* from participating in neoplastic progression.

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