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Inhibition of DNA-Dependent Transcription by the Leader RNA of Vesicular Stomatitis Virus: Role of Specific Nucleotide Sequences and Cell Protein Binding

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The leader RNA transcript of vesicular stomatitis virus inhibits transcription of the adenovirus major late promoter and virus-associated genes in a soluble HeLa cell transcription system. We examined the specific nucleotide sequence involved and the potential role of leader-protein interactions in this inhibition of RNA polymerase II- and III-directed transcription. Using synthetic oligodeoxynucleotides homologous to regions of the leader RNA molecule, we extend our previous results (B. W. Grinnell and R. R. Wagner, Cell 36:533–543, 1984) that suggest a role for the AU-rich region of the leader RNA or the homologous AT region of a cloned cDNA leader in the inhibition of DNA-dependent transcription. Our results indicate that a short nucleotide sequence (AUUAUUA) or its deoxynucleotide homolog (ATTATTA) appears to be the minimal requirement for the leader RNA to inhibit transcription by both RNA polymerases, but sequences flanking both sides of this region increase the inhibitory activity. Nucleotide changes in the homologous AT-rich region drastically decrease the transcriptional inhibitory activity. Leader RNAs from wild-type virus, but not from a 5′-defective interfering particle, form a ribonuclease-resistant, protease-sensitive ribonucleoprotein complex in the soluble HeLa cell extract. Several lines of evidence suggest that the leader RNA specifically interacts with a 65,000-dalton (65K) cellular protein. In a fractionated cell extract, only those fractions containing this 65K protein could reverse the inhibition of DNA-dependent RNA synthesis by the plus-strand vesicular stomatitis virus leader RNA or by homologous DNA. In studies with synthetic oligodeoxynucleotides homologous to leader RNA sequences, only those oligonucleotides containing the inhibitory sequence were able to bind to a gradient fraction containing the 65K protein.

Vesicular stomatitis virus (VSV) is a highly virulent virus that interferes with various metabolic functions in the host cell (40). A principal function that is affected by VSV is cellular transcription (4, 41). The ability of VSV to inhibit cellular RNA synthesis was shown to be dependent on primary transcription of the genome (43, 45). However, studies with UV-inactivated virus have demonstrated that only a small portion of the 3′ end of the genome needs to be transcribed to obtain the inhibitory activity (42). Since the VSV genome has been shown to be transcribed sequentially from its 3′ end (1, 3), it has been suggested that the first viral transcript, a 47-nucleotide leader RNA, was the inhibitor molecule (42). Experiments by Kurilla et al. (21) have demonstrated that the leader RNA is present in the nucleus of cells infected by VSV, and studies by McGowan et al. (28) have demonstrated that purified leader RNA (Indiana serotype) can inhibit DNA-dependent transcription of adenovirus and simian virus 40 genes in a soluble whole-cell extract. We recently have demonstrated that the inhibition of transcription in the infected cell correlates with the ability of the virus to transcribe its leader RNA, and by kinetic studies we have also demonstrated a direct correlation between the level of leader RNA production and the degree of cellular RNA synthesis inhibition (13). These earlier studies provide strong evidence that the leader RNA transcript of VSV is at least partially responsible (45) for inhibiting host cell RNA synthesis.

The ability of VSV to shut off cellular RNA synthesis appears to be at the level of transcription initiation, probably by preventing binding of the enzyme to its template (44). There is no evidence for altered posttranscriptional modification events (41), nor is there evidence for enhanced degradation of cell mRNA (29). However, it is not clear how the leader RNA might be interacting with the transcriptional machinery to block initiation. In infected cells, Kurilla and Keene (20) have found that VSV leader RNA can be immunoprecipitated with antisera against the La protein, which may be an RNA polymerase III transcription factor, and is bound to *pol*III precursor RNA (31). However, the relationship between the binding of the leader RNA to the cellular La protein and the inhibition of transcription is not known.

The leader RNAs of both *wt* VSV serotypes and of several strains have been sequenced (6, 7, 12, 18) and all contain an AU-rich central region (see Fig. 1). In contrast, the leader RNA of the VSV defective-interfering (DI) particle, which does not inhibit cellular transcription (13, 28), does not have this AU sequence (34; see Fig. 1). Earlier experiments demonstrated that a ribonuclease T1 fragment of the leader RNA that contained the AU-rich sequence inhibited transcription of both the *pol*II (adenovirus type 2 [Ad2] late promoter [LP]) and *pol*III (Ad2 adenovirus-associated [VA] genes) in an in vitro system (14). In addition, the secondary structure in the leader RNA of the VSV New Jersey serotype (VSV_{NJ}), that included the AU region, appeared to be responsible for the enhanced inhibitory activity of this leader molecule. Thus, the AU region appeared to be involved. While somewhat surprising, a VSV cDNA homologous to the leader RNA sequence also inhibited transcription, and furthermore, this DNA leader molecule displayed

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the same dose-response patterns for the inhibition of the LP and VA genes as was observed for the leader RNA itself. This suggests that the cDNA leader functions in the same manner as does the leader RNA and serves as an appropriate model. Although a synthetic oligodeoxynucleotide containing the AU region homolog displayed inhibitory activity, the exact sequence involved was not defined nor was the role of sequences flanking this AU(AT) region.

From the results of these previous studies it is apparent that the leader RNA or homologous DNA sequences are capable of interacting in some way with the cellular transcription apparatus, thereby preventing the initiation of DNA-dependent trancription. Thus, the purposes of the studies reported here were (i) to further define the nucleotide sequence in the leader molecule required for its ability to inhibit transcription; (ii) to determine if the leader RNA interacts with cellular constituents; and (iii) to assess which cellular constituents might be the putative target(s) for the transcriptional inhibitory activity of the VSV leader RNA. Based on studies using homologous deoxynucleotides, we demonstrate that a short palindromic sequence (ATTATTA) appears to be required to obtain inhibition of both RNA polymerase II- and III-directed transcription by the leader sequence. We show that the wild-type leader RNA of both VSV New Jersey and Indiana (VSV_{Ind}), but not of VSV_{Ind} DI-T, interact with a cellular protein(s) to form a high-molecular-weight complex. This interaction appears to involve binding to a 65,000-dalton (65K) cellular protein and, based on results with the leader homologs, to be sequence specific. A cellular fraction containing this protein could prevent the leader RNA from inhibiting both RNA polymerase II- and III-directed transcription.

MATERIALS AND METHODS

Viruses and cells. The VSV used in this study were the San Juan strain of VSV_{Ind} originally obtained from L. O. Mott, U.S. Agricultural Research Center, Beltsville, Md.; the Hazelhurst strain of VSV_{NJ} obtained from the American Type Culture Collection (ATCC 159); and VSV_{Ind} DI-T obtained from S. U. Emerson, University of Virginia. The viruses were grown and purified as previously described (14). BHK and L cells were grown as described by McAllister and Wagner (27). HeLa cells were grown in Dulbecco modified Eagle Medium, which was supplemented with 5% calf serum and 10% tryptose-phosphate broth (GIBCO Laboratories, Grand Island, N.Y.)

Synthesis and purification of the VSV leader RNA transcript. The VSV leader RNAs were produced in an in vitro transcription system (11) under conditions previously described (14). Leader RNA was purified by electrophoresis on 8 M urea–20% polyacrylamide gels in buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA, and 5 mM boric acid at 1,600 V. The leader RNA, labeled with [^{32}P]UMP and detected by autoradiography, was eluted from the gel overnight in buffer containing 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. Acrylamide fragments were removed by filtration of the eluate through a 0.22-μm Milex-GV filter unit, and the leader RNA was precipitated by the addition of 3 volumes of ethanol. The precipitate was collected by centrifugation at 12,000 × *g* for 30 min and was suspended in 1× SET buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 8.0]). Following one extraction with phenol and two extractions with chloroform-isoamyl alcohol (24:1 [vol/vol]), the leader RNA was repeatedly precipitated with ethanol (at least three times), and the final RNA pellet was suspended in 10 mM Tris hydrochloride (pH 8.0). The amount of leader RNA was determined both by relative incorporation of UMP and by optical density at 260 nm.

DNA-dependent trancription with a HeLa cell extract. A transcriptionally active extract was prepared from HeLa cells as described by Manley et al. (24). The DNA templates used for transcription were the pBR322-Bal-1E recombinant of the Ad2 DNA late promoter (originally obtained from P. A. Sharp, Massachusetts Institute of Technology) and a pBR322 recombinant of the VA RNA genes (originally a gift from D. F. Bogenhagen, Carnegie Institute). Both recombinant plasmids were propagated in *Escherichia coli* HB101 and were purified by CsCl gradient centrifugation. Prior to transcription, the Ad2 late promoter was cleaved with the restriction endonuclease *Sma*I in the buffer specified by the manufacturer (Bethesda Research Laboratories).

The DNA templates were transcribed in a reaction mixture containing 3 mM $MgCl_2$; 15 mM ammonium sulfate; 0.1 mM EDTA; 1 mM dithiothreitol; 5% glycerol; 1 mM each of ATP, CTP, and GTP; 0.1 mM UTP containing 10 μCi of [α-^{32}P]UTP (410 Ci/mmol) and 20% (vol/vol) of HeLa cell extract. After 30 min at 31°C, reactions were stopped by adding 10 vol of 1× SET buffer and extracting once with SET-saturated phenol. After 30 μg of yeast carrier RNA was added, the transcription products were precipitated from the aqueous phase by the addition of sodium acetate to 0.3 M and 3 volumes of ethanol. The precipitate was collected by centrifugation at 10,000 × *g* and suspended in 8 M urea containing 0.1% bromophenol blue and xylene cylanol, and the transcription products were separated on 8 M urea–8% polyacrylamide slab gels in the Tris-borate buffer described above.

Purification and sequencing of oligodeoxynucleotides. Oligodeoxynucleotides of defined sequence were synthesized by either phosphoramidite chemistry (kindly provided by E.-L. Winnacker, Institute of Biochemistry, University of Munich) or phosphite chemistry (kindly provided by M. Smith and J. Fox, Department of Microbiology, University of Virginia). The 5′ blocking group, dimethoxytrityl, was removed from oligonucleotides synthesized by phosphoramidite chemistry by incubating in 80% acetic acid at 20°C for 30 min. After removal of the acetic acid by lyophilization, the oligodeoxynucleotides were suspended in 10 mM Tris hydrochloride (pH 8.0, 1 mM EDTA). The synthetic oligonucleotides were purified on and eluted from a 20% sequencing gel as described above. Samples were further purified by gel filtrations on a Sephadex G100 column equilibrated with 0.3 M ammonium acetate. After repeated lyophilization, the purified oligodeoxynucleotide was suspended in distilled water and stored at −80°C.

For sequence analysis, oligodeoxynucleotides were 5′-end-labeled with [γ-^{32}P]ATP and polynucleotide kinase as described by Maxam and Gilbert (26) and sequenced by the method of Maxam and Gilbert modified as follows: C and C+T reactions were incubated for 20 min and G reactions were incubated for 8 min at 20°C. A+G reactions were incubated for 40 min at 37°C. Prior to ethanol precipitation, 30 μg of yeast tRNA was added.

Fractionation of cell extracts. Sucrose solutions for rate-zonal separations of HeLa cell transcription extracts were prepared in 10 mM Tris (pH 7.8)–3 mM $MgCl_2$–15 mM ammonium sulfate –0.1 mM EDTA–1 mM dithiothreitol–5% glycerol (HSG buffer), and NaCl concentrations ranged from 10 mM to 400 mM. Sucrose gradients (5 to 15%) were

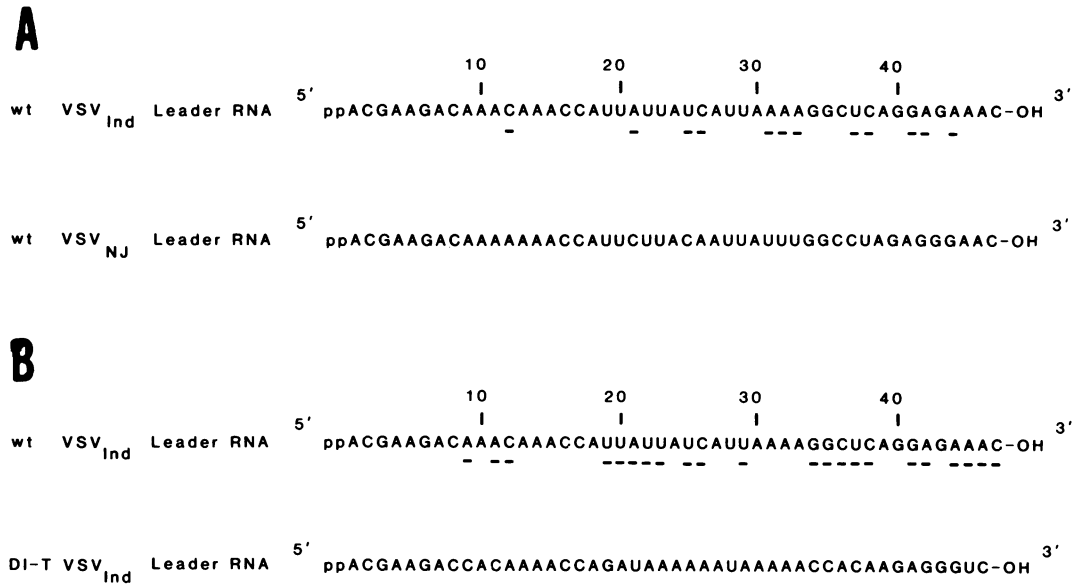

FIG. 1. Comparison of the nucleotide sequences of the leader RNAs of wild-type (*wt*) VSV_{Ind} and VSV_{NJ} (A) and *wt* VSV_{Ind} and DI-T VSV_{Ind} (B). The unique regions of the VSV_{Ind} leader RNA in (A) and (B) are highlighted by underlining. Data are from Colonno and Banerjee (6, 7) and Schubert et al. (34).

centrifuged in an SW60 rotor at 59,000 rpm from 4 to 6 h at 4 to 37°C. The 4S and 18S rRNAs (Bethesda Research Laboratories) were used as sedimentation rate markers. When necessary, gradient fractions were simultaneously dialyzed and concentrated in a Centron 10 microconcentrate as described by the manufacturer (Amicon Corp., Lexington, Mass.).

Metrizamide solutions for isopycnic gradient separations were prepared in HSG buffer containing 0.1 M NaCl. Linear gradients of 15 to 55% were centrifuged to equilibrium in an SW60 rotor at 58,000 for 24 h at 4°C. The density of selected gradient fractions was determined from the refractive index using the calculation derived by Rickwood and Birnie (30).

The molecular weight of leader RNA-protein complexes was estimated by gel filtration chromatography on a Biogel A1.5 matrix (Bio-Rad Laboratories, Richmond, Calif.) in HSG buffer containing 0.1 M NaCl. Size markers used to calibrate the column were ferritin (470K), hemoglobin (64.5K), and myoglobin (16.8K).

Leader RNA binding to immobilized cell proteins. Proteins in the HeLa cell extract were separated on a 12.5% polyacrylamide gel in the buffer system described by Laemmli (22). Separated proteins were transferred from the gel to nitrocellulose (0.1 μM; Schleicher & Schuell Co., Keene, N.H.) by electroblotting (38) at 150 mA for 18 h in buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol. The efficiency of transfer was monitored by staining a portion of the nitrocellulose filter in a solution of 0.1% amido black–50% methanol–10% acetic acid followed by destaining in 90% methanol–5% acetic acid. After transfer, the nitrocellulose filter was incubated for 1 to 2 h at 37°C in a solution containing 5 mg of yeast core RNA (Sigma Chemical Co., St. Louis, Mo.) per ml, 10 mM Tris (pH 7.8), and 150 mM NaCl. The blot was washed two times in 10 mM Tris for 10 to 15 min and was incubated for 3 to 6 h with a ^{32}P-labeled RNA probe in 10 mM Tris (pH 7.8)–1 mM EDTA–50 mM NaCl–0.02% Ficol–0.02% polyvinylpyrollidone–0.02% bovine serum albumin (nuclease-free; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The filter was washed four times in the solution described above containing 0.1 M NaCl and without the labeled probe.

Purified VSV leader RNAs were labeled by ligation of [5'-^{32}P]cytidine-3'-5' bisphosphate to the 3' end by T4 ligase (Bethesda Research Laboratories) in a reaction mixture described previously (13). After overnight incubation at 4°C, the labeled leader RNA was purified by electrophoresis on an 8 M urea polyacrylamide gel and eluted as described above. In the binding experiments described above, the probe concentration was approximately 5×10^5 cpm/ml.

Filter binding assay. The nitrocellulose filter binding assay was a modification of the method of Jones and Berg (17). Filters were prewashed in HSG buffer. Samples of ^{32}P-labeled nucleic acid were mixed with various concentrations of HeLa cell extract and incubated for 10 min at room temperature. When binding activities of different RNA or DNA samples were compared, efforts were made to ensure that equivalent specific activities were used. After incubation, the mixture was diluted in HSG buffer and filtered through a 0.45-μM-pore-size Millipore type HA filter at a flow rate of approximately 5 ml/min. The filters were extensively washed, and the amount of binding was determined by Cerenkov counting.

RESULTS

Effect of oligodeoxynucleotides homologous to leader RNA sequences on DNA-dependent transcription. Shown in Fig. 1 are the nucleotide sequences of the *wt* VSV_{Ind}, *wt* VSV_{NJ}, and VSV_{Ind} DI leader RNAs. As indicated above, previous results have demonstrated that the wild-type leader RNAs of VSV inhibit DNA-dependent trancription from simian virus 40 promoters, the adenovirus major LP and VA genes (13, 28), whereas the DI leader RNA displays little ability to inhibit transcription of these genes. In addition, previous results with RNase T1 fragments have suggested that the AU-rich region (nucleotides 18 to 33), which is not present in the DI leader, might be important in the inhibitory activity of the wild-type leader RNA (14). Since a homologous DNA leader also has been shown to inhibit transcription, one

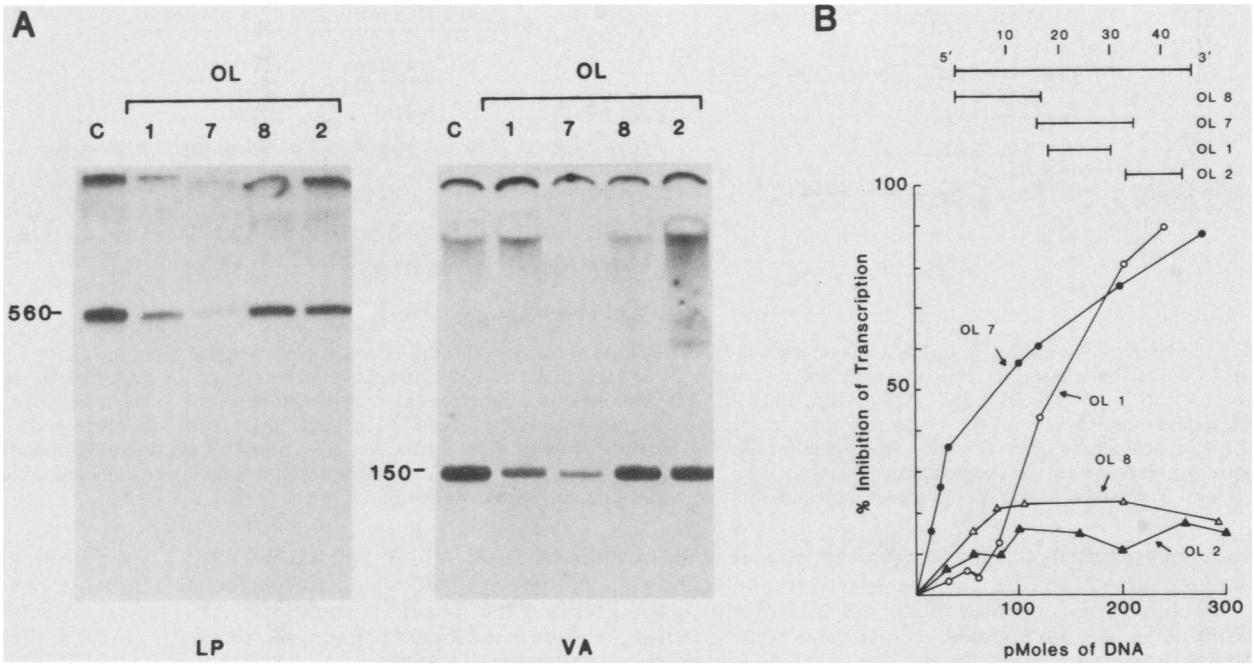

FIG. 2. DNA-dependent transcription of the adenovirus LP and VA genes and the comparative inhibition of transcription by synthetic oligodeoxynucleotides homologous to various regions of the *wt* VSV_{Ind} leader transcript. The VA gene and LP gene pBR322 recombinants (37 ng/μl; concentration, 10^{-8} M) were incubated in the HeLa cell extract alone or with added synthetic oligodeoxynucleotides (OL8, OL7, OL1, and OL2) homologous to the various regions of the leader molecule as indicated in (B). [^{32}P]UMP-labeled transcription products were separated on 8 M urea–8% polyacrylamide gels. (A) Autoradiographs of VA gene transcription products showing the level of the 150-base transcript in the presence of 150 pmol of the indicated oligodeoxynucleotides and LP gene transcription products showing the level of the 560-base transcript in the presence of 280 pmol of the indicated oligodeoxynucleotides. The respective control (C) levels of transcription of each gene are shown. (B) Dose response to the inhibition of VA gene transcription by the indicated oligodeoxynucleotides. The extent of inhibition was determined by dividing the amount of transcription of each template in reactions containing the oligodeoxynucleotide by the amount in control reactions with no added oligodeoxynucleotide. The amount of transcription was determined by Cerenkov counting of the specific transcripts eluted from gel slices.

approach for further defining the sequence and specific nucleotides involved in inhibiting RNA polymerase II- and III-directed transcription was to synthesize synthetic oligodeoxynucleotides representing most regions of the leader molecule and to test their effects on DNA-dependent transcription.

In Fig. 2 we show the ability of several synthetic oligodeoxynucleotides to inhibit transcription in a reconstituted HeLa cell system (24) with the cloned Ad2 LP and VA genes as templates. The location of each oligodeoxynucleotide relative to the *wt* VSV_{Ind} leader sequence is indicated. The addition of an oligodeoxynucleotide (OL) to the in vitro transcription reaction, corresponding to leader nucleotides 18 to 30 (OL1), reduced the level of both the 150-nucleotide VA and 560-nucleotide LP gene transcripts (Fig. 2A) when compared with the respective controls (labeled C). However, an oligodeoxynucleotide corresponding to leader nucleotides 16 to 35 (OL7) reduced the level of transcription from both templates to a greater extent than did OL1. OL8 (leader nucleotides 1 to 17) and OL2 (nucleotides 33 to 44) representing the 5′ and 3′ regions of the leader molecule, respectively, showed little inhibitory activity when added to the in vitro transcription reactions. In these experiments 150 pmol of each oligodeoxynucleotide was added to the VA gene transcription reactions, and 280 pmol of each was added to LP gene transcriptions. As we have reported previously (13, 14), LP gene transcription is less sensitive than VA gene transcription to the inhibitory effect of leader sequences; more of each inhibitory nucleotide sequence was used in LP gene transcription reactions to obtain equivalent levels of inhibition.

In the experiment described above, the effect of each inhibitory molecule was tested at only one concentration. In Fig. 2B, we show the dose response to the inhibition of VA gene transcription by each indicated DNA molecule. The overall pattern for LP gene inhibition was essentially the same (data not shown). Transcription of the VA gene was performed in the presence or absence of various concentrations of each oligodeoxynucleotide, the transcription products were separated by gel electrophoresis, and the amount of the specific 150-nucleotide transcript was quantitated. At all concentrations tested, OL8 and OL2 displayed low inhibitory activity, which did not increase significantly with increasing doses of these oligodeoxynucleotides. While OL1 showed significant inhibitory activity only above a dose of 100 pmol, OL7, which includes the OL1 sequence, inhibited transcription even at the lowest doses tested. It should be noted that these synthetic oligonucleotides were approximately two- to threefold less effective at inhibiting transcription compared with the full-length leader molecule itself (data not shown).

A quantitative comparison of the inhibitory activities of oligodeoxynucleotides representing various regions of the leader molecule was determined from multiple dose-response curves and is presented in Fig. 3 as the 50% inhibitory dose. OL8 and OL2, corresponding to the 5′ and 3′ ends, respectively, of the leader molecule, exhibited little ability to inhibit transcription with increasing doses. At the

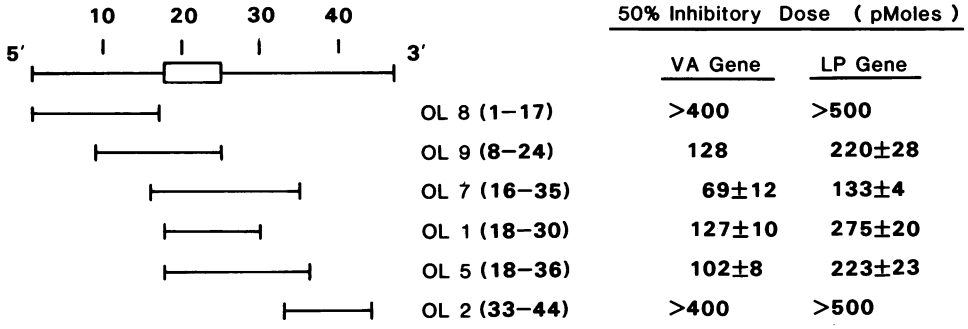

FIG. 3. Comparative inhibition of RNA polymerase II- and III-directed transcription by oligodeoxynucleotides homologous to various regions of the leader transcript. Oligodeoxynucleotides used in these experiments, representing various regions of the leader molecule, are designated 5′ to 3′ as OL8, OL9, OL7, OL1, OL5, and OL2. The 50% inhibitory dose for each oligodeoxynucleotide was determined from dose-response inhibition curves for VA and LP gene transcription as described in the legend to Fig. 2. Each value is the average ± the error of the mean for multiple determinations. The value for the 50% inhibitory dose of OL9 for the VA gene transcription was determined from a single dose-response curve. The numbers in parentheses after each oligodeoxynucleotide number represent the inclusive homologous leader nucleotides. The boxed region on the leader molecule represents the common region shared by OL9, OL7,OL1, and OL5.

highest quantity tested, 400 and 500 pmol added to reactions transcribing the VA and LP genes, respectively, only 20 to 30% inhibition was observed (data not shown); a 50% inhibitory level was not attained even at these high concentrations. The four additional oligodeoxynucleotides shown in Fig. 3 (OLs 1, 5, 7, 9) all inhibited the transcription of both adenovirus genes, with OL7 (nucleotides 16 to 35) being the most efficient. These inhibitory oligonucleotides contain all or part of the AT-rich region previously suggested to be involved in the transcriptional inhibitory activity of the leader molecule (14). The common leader region shared by these oligodeoxynucleotides (residues 18 to 24) is the palindromic sequence ATTATTA. Certain flanking nucleotides may also be important. For example, the amount of OL7 required to achieve 50% transcription inhibition of both genes was approximately 1.5 to 2 times less than that required by the other inhibitory oligodeoxynucleotides. This molecule has nucleotides 5′ and 3′ to the apparent minimal inhibitory sequence ATTATTA. OL9, OL1, and OL5 also have additional nucleotides either 5′ or 3′ to this region, but not flanking on both the 5′ and 3′ sides. This may indicate that although the sequence ATTATTA is the minimal region required for transcriptional inhibitory activity, nucleotides flanking each side of this palindrome are required for maximal inhibitory activity.

We also examined the inhibitory activity of a synthetic oligodeoxynucleotide representing nucleotides 16 to 35 of the VSV_{NJ} leader molecule (Fig. 1, sequence CCATTCTTACAATTATTTGG). This molecule covers the same region as VSV_{Ind} OL7 (Fig. 3). The transcriptional inhibitory activity of this homologous VSV_{NJ} DNA sequence was essentially the same as that observed with VSV_{Ind} OL7 (data not shown).

Effect on transcription inhibition of nucleotide substitutions in the AT region of the homologous leader. The best way to confirm the importance of specific nucleotides in the biological function of a molecule is to alter the sequence and then examine its effect on biological activity. To this end, the transcriptional inhibitory activities of two synthetic oligodeoxynucleotides containing T to G transversions in OL1 were examined in the in vitro transcription system (Fig. 4). Note that in addition to the palindromic sequence of OL1 (nucleotides 18 to 24) which appears to be required for transcriptional inhibitory activity, the ATT sequence is repeated four times (residues 18 to 29) except for one C residue at position 26. There is also a second partial palindrome ^{24}ATCATTA^{30} (note that T to C is a transition). In another synthetic oligodeoxynucleotide (OL3), G residues replace the first T in each OL1 ATT repeat, which also results in the loss of palindromic symmetry. In OL4, the G to T transversion changes the AT-rich nature of the sequence

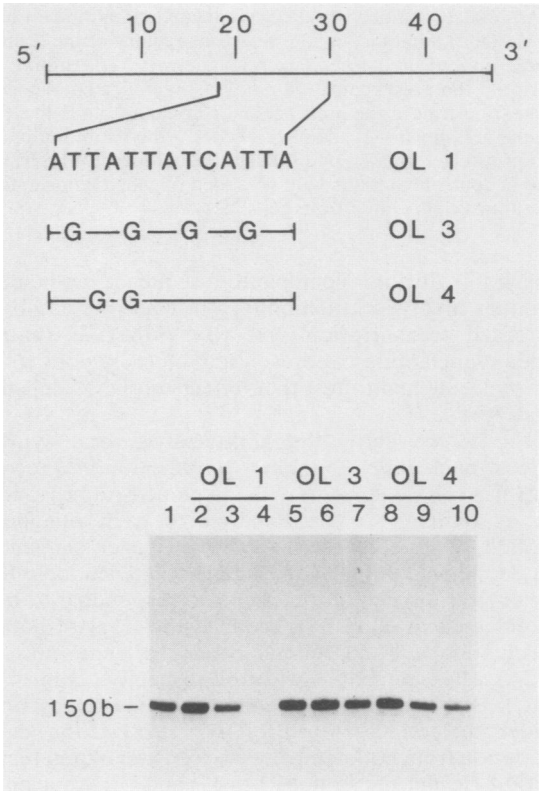

FIG. 4. Effect of nucleotide substitutions in the homologous AT-rich leader sequence of OL1 on the inhibition of VA gene transcription. The pBR322-VA gene recombinant was incubated in the soluble HeLa cell transcription extract alone (control) or with the indicated oligonucleotide sequences. Transcription products were separated on 8 M urea–8% polyacrylamide gels and were visualized by autoradiography. Level of the VA gene transcript: lane 1, alone; lane 2, 30 pmol; lane 3, 120 pmol; lane 4, 240 pmol; lane 5, 55 pmol; lane 6, 165 pmol; lane 7, 400 pmol; lane 8, 50 pmol; lane 9, 180 pmol; lane 10, 390 pmol. b, Base number.

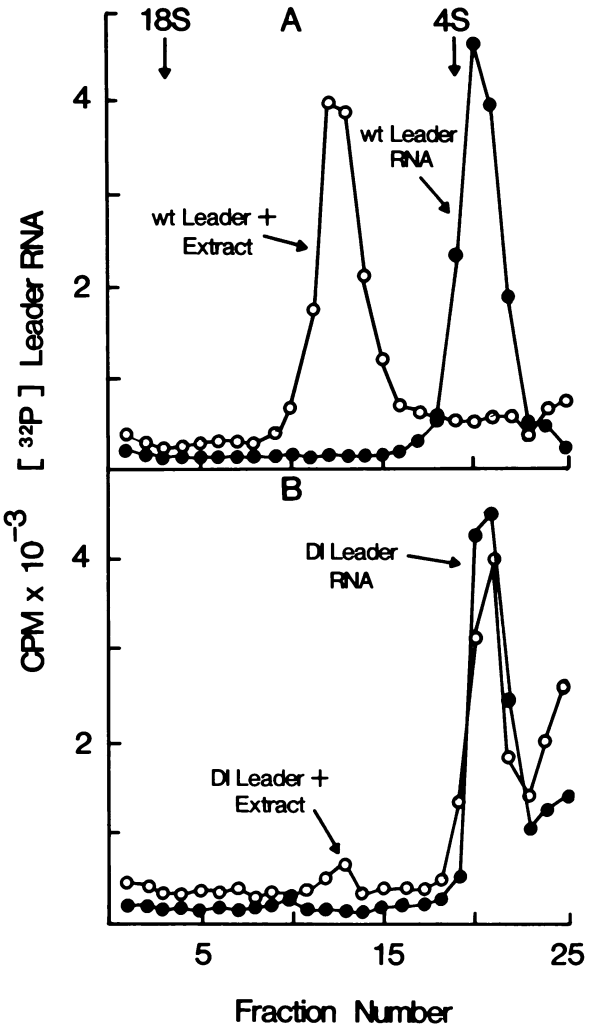

FIG. 5. Sedimentation pattern of ^{32}P-labeled wild-type leader (A) and DI leader (B) RNAs before and after incubation with a soluble HeLa cell extract. Samples of the labeled leader RNAs were centrifuged through a 5 to 25% sucrose gradient alone or after incubation with the HeLa cell transcription extract as described in the text. The position of each leader RNA in the gradient was determined by Cerenkov counting of gradient fractions. The position of rRNA markers in parallel gradients is indicated by arrows.

without eliminating the palindrome. The effect of each oligodeoxynucleotide (OL1, OL3, and OL4) on VA gene transcription was compared, and the data are shown in Fig. 4. Compared with the control level of transcription (lane 1), increasing concentrations of OL1 progressively inhibited transcription as expected (lanes 2 to 4). In contrast, at levels approximately two times those used with OL1, increasing concentrations of OL3 resulted in little reduction in the level of the 150-base VA transcript (lanes 5 to 7). There was some inhibitory activity of VA gene transcription with increasing amounts of OL4 (lanes 8 to 10), but even at levels 1.6 times those used to completely obliterate transcription with OL1 (240 pmol; lane 4), OL4 produced only a partial inhibitory effect (390 pmol; lane 10).

The ability of both OL3 and OL4 to inhibit LP gene transcription also was much reduced compared with that of OL1, and even the marginal inhibitory activity of OL4 observed in VA gene transcription was not observed with LP gene transcription (data not shown). From these experiments it seems apparent that the VSV leader DNA homolog must at least conserve its ATT-rich sequence to retain transcriptional inhibitory activity, and because some ability to inhibit VA gene transcription was retained in OL4, the palindromic symmetry also may play a role in this inhibition.

Interaction of the leader RNA with cellular constituents. Based on previous results (14) and those presented above, the AU region of the leader RNA molecule or AT region in the DNA homolog appears to be sufficient for inhibiting both RNA polymerase II- and III-directed transcription. We next attempted to identify the putative cellular target(s) in the in vitro HeLa cell transcription system. As an initial test to identify any level of interaction between the viral leader RNA and cellular constituents, ^{32}P-labeled leader RNAs were incubated for 5 to 20 min with varying amounts of the HeLa cell extract and centrifuged through a glycerol-sucrose gradient.

The control *wt* and DI leader molecules sedimented at approximately 3.5S relative to rRNA markets (Fig. 5). After incubation of the *wt* leader RNA with the soluble HeLa cell extract, the leader sedimented as a single peak at approximately 8S. In contrast, after incubation with HeLa cell extract, only a small amount of DI leader RNA sedimented at 8S; the majority sedimented as free leader RNA at 3.5S. This demonstrates that the *wt* leader RNA, which inhibits DNA-dependent transcription, associates with a cellular component(s) in the HeLa cell transcription extract to form a high-molecular-weight complex, whereas the DI leader RNA, which does not inhibit transcription (28), shows little capacity to associate with cellular components in this assay. The interaction between the *wt* leader RNA and cellular constituent(s) (Fig. 5A) was independent of the temperature of incubation between 4 and 37°C. In addition, even at 4°C 100% of the leader RNA sedimented as an 8S peak within 5 min of incubation with the transcriptionally active cell extract (data not shown).

The characteristics of the 8S leader complex are summarized in Table 1. The molecular mass of the complex, determined by gel filtration chromatography, was 260K or roughly 15 times that of the free leader RNA. The density of the complex was 1.21 g/ml in metrizamide, a typical value for a ribonucleoprotein complex (30), and the formation of the complex was sensitive to pronase. These data suggest that an integral part of cellular component(s) associated with the

TABLE 1. Properties of the wild-type leader RNA-protein complex recovered from the sucrose gradient shown in Fig. 5

Property	Value
Molecular weight	260,000
Sedimentation coefficient	8S
Density (in metrizamide)	1.21 g/ml
Sensitivity to RNase[a]	−
Sensitivity to protease[b]	+
Salt stability[c]	
VSV_{Ind}	0.15 M
VSV_{NJ}	0.25 M

[a] Determined by sedimentation of the 8S leader complex in 5 to 25% sucrose gradient in the buffer described in the text and containing 1 μg of ribonuclease I (Worthington Diagnostics, Freehold, N.J.) per ml.

[b] Determined by sedimentation of 8S leader complex in a gradient containing 1 μg of pronase (Sigma) per ml.

[c] Determined in sucrose gradients containing various concentrations of NaCl. The value listed is the highest salt concentration in which 100% of the leader RNA sedimented at 8S.

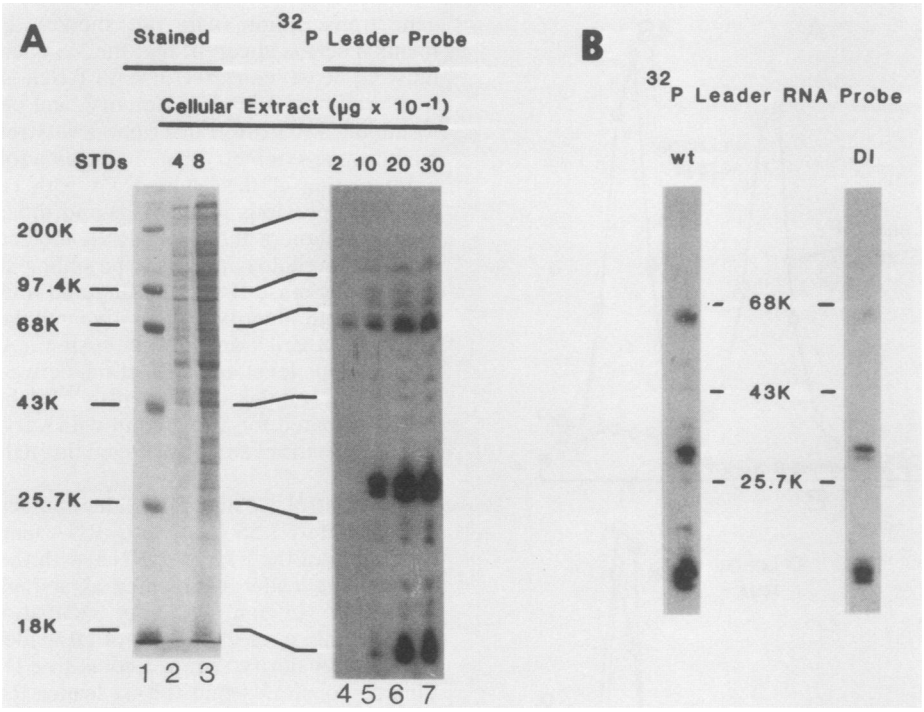

FIG. 6. Detection of binding to HeLa cell proteins by the wild-type and DI VSV_{Ind} leader RNAs. Various amounts of HeLa cell protein were separated on 12.5% polyacrylamide gels, and the proteins were transferred to nitrocellulose and incubated with ^{32}P-labeled leader RNA, as described in the text. (A) Lane 1, protein standards; lanes 2 and 3, two concentrations of HeLa cell extract stained with amido black; lanes 4 through 7, various concentrations of HeLa cell extract blotted onto nitrocellulose and incubated with ^{32}P-labeled *wt* leader RNA probe. (B) Autoradiographs of 100 μg of cell extract blotted onto nitrocellulose and incubated with ^{32}P-labeled *wt* and DI leader RNA probes.

wt leader molecule was protein. In addition, the leader RNA in the 8S peak was resistant to ribonuclease; essentially all of the leader molecule sedimented at 8S when preincubated with the cell extract and sedimented through a gradient containing ribonuclease I. Under the same conditions, the DI leader remained sensitive to ribonuclease (data not shown). By gel electrophoresis we determined that the entire full-length, 47-nucleotide leader sequence was present in the 8S peak after ribonuclease treatment. The 8S complex formed with the VSV_{Ind} leader RNA was stable in 0.15 M NaCl while that formed with the VSV_{NJ} leader RNA was somewhat more stable, dissociating only at concentrations above 0.25 M NaCl. This is interesting because we have previously determined that the leader RNA of the VSV_{NJ} serotype is a more effective inhibitor of DNA-dependent transcription than is the leader RNA of the VSV_{Ind} serotype, and the difference in inhibitory activities appears to be due to RNA secondary structure present in the VSV_{NJ} leader molecule (14). Thus, the more effective inhibitor of transcription formed a more salt-stable complex with components of the cell transcription extract.

We next attempted to identify the specific protein constituents involved. Initial experiments to isolate and purify the 8S complex using successive gradient centrifugation and column chromatography failed because of the instability of the complex under these conditions. Alternatively, we attempted to detect specific proteins that had the capacity to bind to the leader RNA by protein blotting techniques (5). Proteins in the cell extract were separated by gel electrophoresis and transferred to nitrocellulose filters by electroblotting, and the immobilized proteins were incubated with a ^{32}P-labeled *wt* leader RNA probe (Fig. 6). The HeLa cell extract contained a large number of proteins of various sizes as shown in the stained gel (Fig. 6A, lanes 1 to 3). When the proteins in 20 μg of the HeLa cell extract were examined by the protein blotting technique utilizing a *wt* leader RNA probe, a band at 65K was detected (Fig. 6A, lane 4). With increasing concentrations of protein blotted, we observed increased binding of the leader RNA to the 65K protein. It should be noted that the binding conditions that we used, i.e., low salt, low temperature, and incubation times of 5 to 10 min, would not favor annealing of the leader RNA to nucleic acids that might be present in the whole cell extract. Moreover, when blots were treated with pronase prior to incubation with the labeled leader RNA, no binding was observed. These data, along with the pronase sensitivity of the leader RNA complex described in Fig. 5 and Table 1, indicate that the band at 65K bound by the leader RNA is a protein.

With increasing concentration of HeLa cell protein blotted, we observed increasing binding to the 65K protein, but also binding to proteins at ~32K and ~18K. As was shown in Fig. 5, the DI leader RNA does not form a complex to any significant extent with the HeLa cell proteins under physiological conditions. To determine if the binding of the leader RNA to any of the proteins in Fig. 6A was specific, we examined the binding activity of the DI leader RNA as well as heterogeneous yeast core RNA. Using 20 μg of protein, an amount at which we detected only binding to the 65K protein by the *wt* leader RNA, no binding was observed by labeled DI leader RNA or yeast core RNA (data not shown). However, as we increased the concentration of extract, as with the *wt* leader RNA, binding to the 32K and 18K proteins was detected with both DI and yeast RNA controls. Fig. 6B shows a comparison of the protein binding abilities of *wt* and DI leader RNAs with a high concentration of protein blotted (100 μg). Although there was strong binding to the 32K and 18K proteins, very little binding of DI leader to the 65K

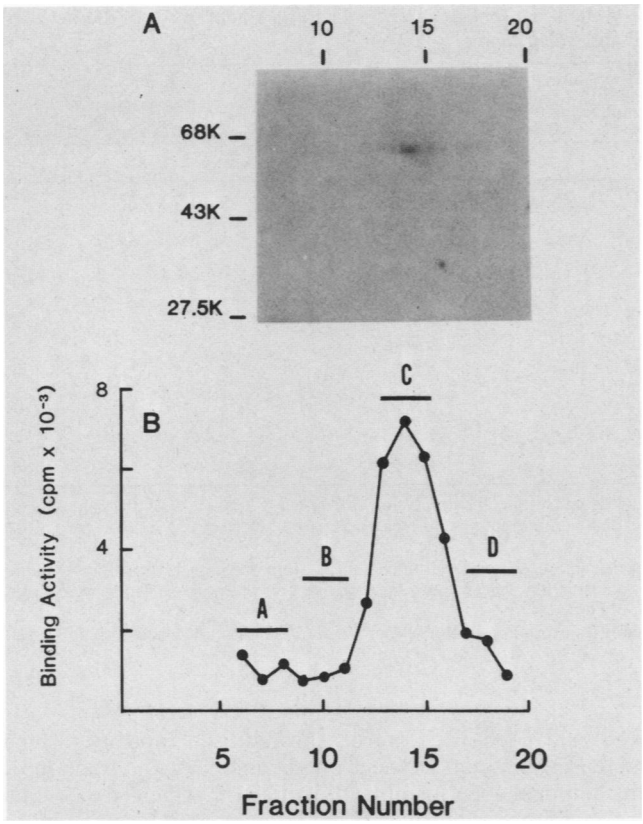

FIG. 7. Binding of the wild-type leader RNA to proteins in a glycerol-sucrose gradient-fractionated HeLa cell extract. HeLa cell protein (100 μg) was sedimented through a 5 to 25% sucrose gradient; fractions were collected, dialyzed, and concentrated to the original volume. (A) Autoradiograph showing position of the 65K binding protein in the sucrose gradient after protein blotting onto nitrocellulose and incubation with a ^{32}P-labeled leader probe. Protein size markers are indicated. (B) Ability of each gradient fraction to bind with the ^{32}P-labeled VSV_{Ind} leader RNA in the filter binding assay. Each fraction was incubated with ~10^4 cpm of leader RNA for 7 min prior to filtration.

protein was detected. Relative to the levels of binding to the 32K and 18K proteins, there was 7- to 10-fold less binding to the 65K protein by the DI leader RNA than by the *wt* leader RNA, even at these high concentrations of cell protein. In all experiments, efforts were made to ensure that the specific activities of the probes were equivalent. Thus, the *wt* leader RNA sequence but not the DI sequence bind efficiently to a 65K cellular protein, whereas both leader RNAs as well as yeast core RNA bind to equivalent levels to other, likely nonspecific nucleic acid binding proteins, as the concentration of protein blotted is increased. Although the *wt* and DI leader RNAs have similar sequences, the inhibitory AU sequence is missing in the DI leader, suggesting that these sequences may be involved in the binding to the 65K protein. Further evidence for this suggestion was obtained in the experiments described below.

Binding of the leader RNA to a fractionated HeLa cell extract. To further examine the binding activity of the leader RNA, samples of the HeLa cell extract were sedimented through a glycerol-sucrose gradient; fractions were collected, dialyzed, and concentrated as described in Materials and Methods. The proteins in each fraction were separated by gel electrophoresis and transferred to nitrocellulose, and the immobilized proteins were incubated with a ^{32}P-labeled leader RNA probe. In addition, the ability of leader RNA to bind to components in each fraction was assessed by a filter binding assay.

When each cell extract fraction was examined for blotting by ^{32}P-leader RNA, only the 65K protein could be detected (Fig. 7A, fractions 13 to 15); binding of the leader RNA to the apparent general nucleic acid-binding proteins at 32K and 18K was not observed, as in previous experiments with the whole-cell extract (Fig. 6A). The failure of the ^{32}P leader to react with 32K and 18K proteins after gradient fractionation of the HeLa cell extract suggests that, unlike the 65K protein, these proteins are not concentrated in any one peak in the gradient. Relative to the rRNA markers, the 65K protein sedimented at ~7S to 8S or at an apparent molecular weight of ~240K to ~260K; therefore, in the absence of the leader RNA, the 65K protein appears to be present in the cell extract as a complex roughly four times its molecular weight. In the presence of the leader RNA, the ribonucleoprotein complex sedimented at ~8S or ~260K, i.e., roughly the mass of the leader RNA plus a 240K tetramer (Fig. 5).

We further examined and quantitated the leader binding activity in each gradient fraction with a modification of the nitrocellulose filter binding assay described by Jones and Berg (17). The labeled leader RNA was incubated with each

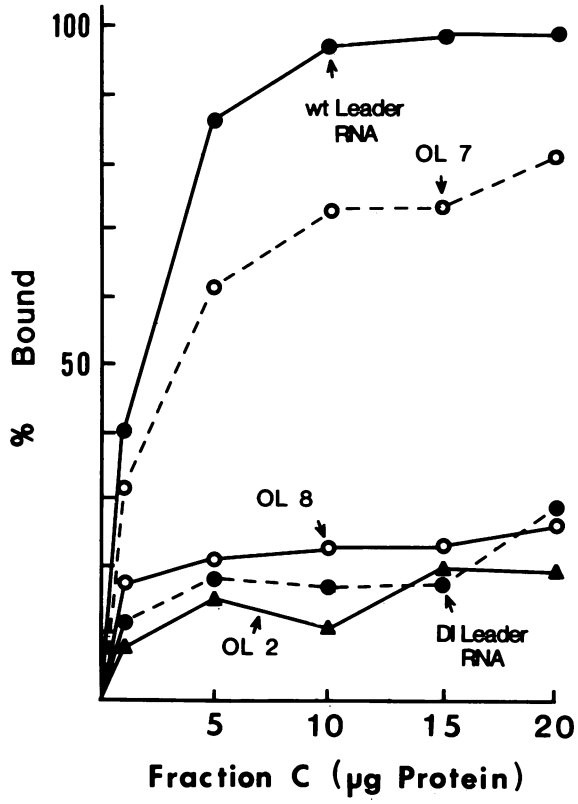

FIG. 8. Comparative binding abilities of VSV leader RNAs and synthetic oligodeoxynucleotides to increasing amounts of HeLa cell fraction C shown in Fig. 7B. ^{32}P-labeled *wt* VSV_{Ind} leader RNA; VSV DI-T (DI) leader RNA; and OL7, OL8, and OL2 (see Fig. 3 for nucleotide sequences) were incubated with the fraction C cellular extract; and the RNA-protein complexes were isolated by the nitrocellulose filter binding assay (17) as described in the text. The percentage of RNA bound was determined by dividing the number of Cernekov counts trapped on the nitrocelluose filters by the number of counts incubated with the fraction C extract.

TABLE 2. Effect of the HeLa cell gradient fractions shown in Fig. 7B on the inhibitory action of *wt* VSV_{Ind} leader RNA in the in vitro transcription of Ad2 LP and VA genes[a]

HeLa cell supplement in transcription reaction[b]	Leader RNA[c]	Amt of ^{32}P (cpm) in the following transcripts:		% inhibition of transcription in the following transcripts:	
		VA	LP	VA	LP
None	−	3,407	2,453		
	+	859	796	75	68
Unfractionated cell extract	−	3,509	2,653		
	+	1,147	953	67	64
Fraction A	−	3,307	2,501		
	+	975	742	71	70
Fraction B	−	3,640	2,574		
	+	1,208	899	67	65
Fraction C	−	3,582	2,350		
	+	2,845	1,842	21	22
Fraction D	−	3,343	2,331		
	+	1,184	798	65	66

[a] *In vitro* transcription reactions were carried out in standard reaction mixtures containing HeLa cell extracts and Ad2 LP or VA genes with or without added VSV *wt* leader RNA. Each reaction mixture was not supplemented or was supplemented with unfractionated HeLa cell extract or fractions A, B, C, or D from the glycerol-sucrose gradient shown in Fig. 7B.

[b] The amount of HeLa cell protein added was 15% of the total reaction volume. Each fraction was adjusted to contain the same volume as the original volume of whole-cell extract layered and separated on the gradient. Thus, the proteins in each fraction were in the same relative concentrations as they were in the unfractionated extract.

[c] A sufficient amount of *wt* leader RNA was added to achieve 65 to 75% inhibition of transcription from each template (55 pmol for VA and 95 pmol for LP DNA).

fraction of the HeLa cell extract, the protein nucleic acid complexes were isolated on nitrocellulose filters, and the level of binding was determined as described in Materials and Methods. The ability of each gradient fraction to bind to labeled leader RNA revealed that maximal activity peaked around fraction 14 (Fig. 7B), corresponding to the exact location of the 65K protein peak (Fig. 7A). The gradient used in this experiment was identical to that used to isolate the 8S leader RNA complex (Fig. 5). Thus, the filter binding assay correlates with the protein blotting assay and suggests that the 65K protein or part of the 8S complex observed under physiological conditions is involved. For further analysis, fractions encompassing the peak of binding activity containing the 65K protein were pooled (designated fraction C) as were several other portions of the gradient as control samples.

Protein binding activity of the synthetic oligodeoxynucleotides homologous to the leader RNA. As indicated above, the DI leader RNA does not inhibit transcription and does not contain the AU region that apparently is required in the *wt* sequence for transcription inhibitory activity. In view of the differential ability of the *wt* and DI leaders to bind the 65K protein, an attempt was made to assess the role of specific sequences in this binding activity by examining the ability of leader homologs OL2, OL7, and OL8 to bind to fractions of the whole-cell extract, specifically to fraction C containing the 65K protein. OL2 and OL8 represent the 3′ and 5′ leader sequences, respectively, whereas OL7 contains the AT sequence required for transcriptional inhibitory activity (Fig. 3). When gradient fractions A, B, and D (Fig. 7B) were examined, no significant binding could be observed with any of the oligodeoxynucleotides. However, with an increasing concentration of fraction C, which contained the 65K binding protein, a dose-responsive binding to OL7, as well as the *wt* leader RNA, was observed (Fig. 8). Although OL7 did not bind to the same level as the *wt* leader RNA, the overall dose-response pattern was the same. As indicated above, it also takes more OL7 than *wt* leader RNA to obtain the same level of inhibition. OL2, OL8, and the DI leader RNA, included as a control, showed little capacity to bind increasing amounts of the protein components in fraction C. In additional experiments, OL5 and OL9, which also contain the inhibitory sequences (Fig. 3), demonstrated binding to fraction C (data not shown). In protein blotting experiments with OL2, OL8, and OL7 as probes, only OL7 displayed detectable but weak binding to the 65K protein (data not shown). Although the level of binding was less than that observed with the *wt* leader RNA, the above data support the contention that the inhibitory and binding sequences overlap.

Effect of HeLa cell extract fractions on transcription inhibition by the *wt* leader RNA. The data presented above and previously (14) indicate that the short AU (AT) regions of leader RNA and homologous DNA sequences are responsible both for inhibiting DNA-dependent transcription, and data from the homologous DNA studies suggest that these same sequences may be involved in binding to the 65K cellular protein. If the nucleotide sequences of the *wt* leader RNA responsible for transcription inhibition are the same as those that bind the 65K HeLa cell protein, then excess HeLa cell fractions rich in the 65K protein might be expected to prevent transcription inhibition by VSV *wt* leader RNA.

In Table 2 we show the results of experiments in which the gradient fractions of HeLa cell extract described in Fig. 7 were tested for their ability to prevent the leader RNA sequence from inhibiting transcription of the VA and LP genes. These gradient fractions were added to standard transcription reactions containing the inhibitory VSV_{Ind} *wt* leader RNA. The amount of leader RNA used in the reactions was chosen to provide ~75% inhibition of transcription from each gene in assays not supplemented with gradient fractions. When the gradient fractions were examined in this way, we found that only fraction C, which contained the 65K binding protein, was able to prevent the inhibition by *wt* leader RNA of polymerase II (LP) and polymerase III (VA) transcription. When each fraction was added to transcription reactions not containing the leader sequence, very little effect on transcription (±5 to 10%) was observed by any

fraction. Also, the addition of purified RNA polymerase II to control or leader-inhibited transcription reactions had little effect (data not shown). It is interesting that the addition of unfractionated cell extract to the reaction did not give relief of inhibition when fraction C did. This suggests the possibility of an inhibitor in the total cell extract that is separated away by gradient fractionation. The data shown are for one concentration (15% of total reaction volume) of the added fraction. Addition of slightly higher amounts of HeLa cell fractions (up to 20% of total) had little additional effect. However, when larger volumes of each fraction or the whole-cell extract were added, a loss in specific transcription began to be observed. This effect was observed in the original report on transcription with the whole-cell extract by Manley et al. (24).

Overall, our data suggest that the leader RNA can bind to a 65K cellular protein that is normally present as a high-molecular-weight complex (240,000) in the HeLa cell extract. Because gradient fractions that contain the 65K leader-binding protein can prevent the sequence-specific inhibition of transcription (Table 2), it seems likely that this protein may be, at least in part, involved in the transcription process that is inhibited by VSV *wt* leader RNA. Not shown are similar results in which sucrose-gradient fractions containing the 65K HeLa cell protein also reversed the transcriptional inhibitory activity of the synthetic OL7, which is the DNA homolog of the VSV *wt* leader sequences apparently responsible for transcription inhibition.

DISCUSSION

The small leader RNA transcript of wild-type VSV is capable of rapidly blocking in vitro DNA-dependent transcription (13, 14, 28) and shutting off RNA synthesis in infected host cells (13, 42). Since the VSV leader RNA appears to act by inhibiting initiation of transcription (44), it may provide an interesting system to examine the molecular events in regulation of eucaryotic transcription. Along with our previous results (14) we indicate that a specific AU(AT)-rich sequence is apparently sufficient for the ability of the leader molecule to inhibit transcription of the Ad2 VA genes and runoff transcription of the AD2 LP gene. We found that the leader molecule, apparently through the same sequences, binds to a 65K cellular protein in a HeLa cell transcription extract, and fractions containing this protein could prevent the sequence-specific inhibition of transcription by the VSV leader.

Utilizing synthetic oligodeoxynucleotides homologous to regions of the leader molecule, we confirmed that the AU(AT)-rich region was important for the transcriptional inhibitory activity, and by making base substitutions we arrived at the hypothesis that a palindrome ATTATTA (nucleotides 18 to 24) was involved (Fig. 4). Although the base changes shown in OL3 (Fig. 4) essentially eliminated the ability of this oligodeoxynucleotide to inhibit transcription, the two base changes in OL4, which maintain a palindromic structure, resulted in an oligodeoxynucleotide that retained some ability to inhibit at least VA gene transcription. Since flanking sequences apparently are also important (14; Fig. 3), these experiments would not rule out retention of inhibitory activity due to the AT-rich nucleotides 3′ to the palindrome at nucleotides 18 to 24 or possibly to the second partial palindrome at nucleotides 24 to 30. In any case, the 7 nucleotide repetitive leader sequence from nucleotides 18 to 24 appears to be sufficient for the inhibitory activity of the homologous synthetic leader molecule. Although it seems clear that the AT-rich nature of the leader sequences must be maintained for transcription inhibition, we can only speculate at this time as to the exact order of nucleotides in this region and the role of flanking sequences in the ability to enhance the inhibition of DNA-dependent transcription.

This central AU(AT)-rich region of the leader molecule has been suggested by Rose and Iverson (32) to resemble the TATA homology or Goldberg-Hogness box (2, 15, 16), which is presumably involved in the transcriptional initiation events of RNA polymerase II. While one might conceive of a competitive inhibition of polymerase II-directed transcription by the VSV leader molecule, i.e., mimicking a polymerase II regulatory sequence, the lack of the TATA homology in polymerase III gene regulatory elements would suggest an alternate mechanism. In fact, the AU(AT) region of the leader molecule inhibits polymerase III-directed transcription of the VA gene even more efficiently than it inhibits polymerase II-directed transcription of the LP gene (14). In considering a mechanism, it is important that at least 30,000 active polymerase complexes are present in the infected cell (44), but only several hundred copies of the leader RNA are present in cells exhibiting 50% inhibition of total RNA synthesis (13). We calculated that the amount of leader RNA needed to inhibit transcription in the in vitro transcription system is in the same order of magnitude as that needed to inhibit transcription in the infected cell, taking into account the highly concentrated cell extract. Therefore, it would appear that a direct interaction of the leader sequence with polymerase molecules is an unlikely mechanism for inhibiting transcription, as would a direct interaction with the DNA template in vivo. Utilizing the blotting techniques described in Fig. 6, we in fact have demonstrated that the leader does not interact or bind to any significant extent to subunit components of purified mammalian cell RNA polymerase II (data not shown). However, we must be cautious of any interpretations drawn from the in vitro data, as the conditions in the cell and the in vitro transcription system may be quite different.

Our results demonstrate that the *wt* leader RNA of VSV binds to a 65K protein in the HeLa cell transcription extract. Although the leader RNA also bound to additional proteins, by examining the binding activity of the DI leader RNA and the synthetic DNAs homologous to leader RNA, our results suggest that only the binding to the 65K protein is sequence specific. As indicated above, the DI leader RNA served as an appropriate control RNA molecule to identify potential leader-protein interactions related to transcription inhibitory activity because the DI leader has a sequence similar to that of the *wt* leader; but because it lacks the AU-rich region, it does not inhibit transcription (28). By protein blotting, very little binding of leader RNA to the 65K protein could be detected (Fig. 6). In the sucrose gradient (Fig. 5) and filter binding assays (Fig. 8), we also observed little binding activity of the DI leader RNA with any cell fractions, including those containing the 65K protein. It should be noted that the cellular proteins in the blotting experiments were separated on denaturing gels. Although proteins apparently renature with appropriate electrophoretic transfer techniques (K. Gooderham, *in* J. Walker and W. Gaestra, ed., *Techniques in Molecular Biology*, in press), we cannot rule out the possibility of detection of additional protein interaction with the leader RNA under strictly nondenaturing conditions. However, we detected binding activity of the leader RNA under the physiological conditions of the filter binding assay only to gradient fractions in which the 65K protein was detected by blotting.

In a previous report, we demonstrated that the leader of the VSV_{NJ} serotype was a better inhibitor of DNA-dependent transcription than was the leader RNA of the VSV_{Ind} serotype (13), and furthermore, the difference appeared to be due to the presence of a more stable stem loop structure in the VSV_{NJ} leader (14). In view of these results, it is interesting that the leader RNA of VSV_{NJ} also was able to form a more salt-stable 8S complex (Table 1). In addition, the VSV_{NJ} leader RNA exhibited more efficient protein binding activity than did the leader RNA of VSV_{Ind} in the filter binding assay (data now shown). Hence, there appears to be some correlation between protein binding efficiency and inhibitory efficiency. We also demonstrated that an oligodeoxynucleotide of the VSV_{NJ} leader (nucleotides 16 to 36), without apparent secondary structure, inhibited transcription with the same efficiency as its counterpart sequence in the VSV_{Ind} leader (OL7). A palindromic sequence (AUUCUUA) was also present in the VSV_{NJ} sequence and in the same location as the apparent inhibitory and palindromic sequence of VSV_{Ind}. In the VSV_{NJ} leader RNA, the AUUCUUA sequence would be located on the stem of the proposed stem loop structure (14).

Using synthetic oligodeoxynucleotides, we demonstrated that only those sequences that were able to inhibit transcription displayed cell protein binding activity. While suggestive, the fact that the protein binding and inhibitory sequences appear to be the same does not provide for a causative relationship between the binding and inhibitory activities of this leader RNA. However, the fact that the gradient fraction that contained the binding activity (and the 65K protein) could prevent the leader RNA or its homolog OL7 from inhibiting DNA-dependent transcription appears to support the hypothesis that these activities are related and that the 65K protein bound by the leader RNA may be at least in part involved in DNA-dependent transcription in the in vitro system. However, we must be cautious in our interpretation of the role this protein might play in transcription regulation. Until the 65K protein is purified by homogeneity, we cannot say with certainty that it is the component in fraction C that is responsible for reversing transcription inhibition by the leader RNA. By using a specific antiserum, preliminary experiments indicate that the 65K protein is not related to the La protein (B. W. Grinnell, unpublished data) which previously has been shown to associate with the leader RNA in the infected cell (20).

Certain cell factors required for accurate initiation of in vitro transcription by both polymerase II (9, 10, 25, 33, 39) and polymerase III (19, 35, 37) have been described, and in several cases purified protein factors have been identified (23, 36). It is not clear what, if any, relationship exists between the known transcription factor activities and the 65K protein described in this report. Simply based on size similarity, it is interesting that a 67K factor that stimulates polymerase II transcription in developing shrimp embryos has been identified (8). However, transcription by polymerases II and III is not thought to involve a common factor. In view of the current state of knowledge, it is difficult to accept that a single oligonucleotide sequence could interact with a cellular factor and inhibit transcription by both polymerases; but the leader RNA, apparently via its AU(AT) inhibitory region, does bind to a 65K cellular protein present in a fraction that can prevent the inhibition of transcription of both polymerase II and III genes by the leader RNA or synthetic DNA homologs. Clearly, questions remain to be answered, as is generally true for most aspects of eucaryotic transcription control. Until the process of eucaryotic transcription control is better understood, the mechanism by which the VSV leader RNA inhibits transcription will be difficult to determine. On the other hand, further studies on the inhibition of DNA-dependent transcription by the VSV leader RNA may provide a means of probing a specific step(s) in the transcription process.

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