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E2F from Adenovirus-Infected Cells Binds Cooperatively to DNA Containing Two Properly Oriented and Spaced Recognition Sites

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E2F is a sequence-specific DNA-binding factor which binds to sites that occur in pairs upstream of the adenovirus E1A and E2 early transcriptional start sites. Substantial quantities of E2F activity were found in uninfected-cell extracts, and there was a modest increase in E2F activity during an adenovirus type 5 (Ad5) infection. In uninfected cells, E2F was found to exist in multiple forms that could be separated chromatographically. Extracts prepared at 24 h after Ad5 infection contained a new form of E2F. This infection-specific form may have been a modified version of one of the forms present in uninfected cells. The infection-specific E2F was shown to bind cooperatively to a pair of E2F sites found upstream of the Ad2 early region 2 mRNA cap site. This binding was sensitive to the spacing between the sites and their relative orientation. In contrast, E2F binding in uninfected-cell extracts was unaffected by changes in orientation and spacing, consistent with very low cooperativity or independent binding.

E2F is a DNA-binding factor that was first detected in adenovirus-infected cells by Kovesdi et al. (13). This factor may be involved in the regulation of transcription of two adenovirus early genes, E1A and E2. Both genes have pairs of E2F binding sites upstream of their mRNA cap sites (14). For E1A, these E2F sites are arranged as a direct repeat separated by 63 base pairs (bp). For E2, the E2F sites form an inverted repeat separated by 25 bp. Kovesdi et al. (13) measured E2F binding activity in nuclear extracts by using the two E2F sites from upstream of E2 initiation sites as a substrate for in vitro binding. They observed a dramatic increase in E2F binding activity during an adenovirus infection. This increase in E2F binding requires the function of a 17-kilodalton (kDa) polypeptide encoded by the adenovirus E4-6/7 open reading frame (1, 7a, 19). Yee et al. (21) have purified E2F from infected cells and reported that it stimulates in vitro transcription directed by a promoter containing a pair of E2F binding sites arranged like the sites in the E2 promoter.

We have studied the induction of E2F binding activity during adenovirus infection. By using DNA containing a single E2F site to measure E2F activity by the DNA band shift assay of Fried and Crothers (6), several different forms of E2F in uninfected cell extracts were observed. In extracts made 24 h after infection with adenovirus type 5 (Ad5), we detected a new form of E2F and a modest increase in total E2F binding activity. In contrast, when a DNA probe with the two E2F sites from upstream of the E2 start site was used, a large increase in binding activity in infected-cell extracts was observed. The increase in binding activity was due to cooperative binding of E2F from infected cells at the two E2F sites in the E2 control region. The activity that produced the cooperative complex copurified with the infection-specific activity observed on a single E2F site. Furthermore, since the infection-specific activity and one of the forms from uninfected cells copurified and had the same molecular weight by photoaffinity cross-linking, it appears that the infection-specific form is probably a modified cellular factor.

MATERIALS AND METHODS

Cells and viruses. Spinner cultures of HeLa cells grown in medium containing 10% horse serum were used for all experiments. Infections were performed with wild-type Ad5 (H5wt300 [12]) at a multiplicity of 20 PFU per cell.

Oligonucleotides. Synthetic DNA oligonucleotides were prepared on an automated DNA synthesizer (Applied Biosystems, Inc.), using phosphoramadite chemistry. Since the yield of oligonucleotides up to the size of 35 bases was >90%, no purification other than desalting was performed. The longer oligonucleotides were purified by electrophoresis in a 12% polyacrylamide gel containing 8 M urea. DNA was eluted from the gel and bound to a Sep-Pack column (Whatman, Inc.), from which it was eluted in dimethyl sulfoxide containing 20% H_2O. The eluted material was dried under vacuum; the DNA was dissolved in H_2O and quantified by UV absorbance. The sequences of E1A and E2 single- and double-site probes are presented in Fig. 1A; those of variant E2 probes are listed in Table 1. The sequences of one strand of DNAs used as negative controls are as follow: E1A-zeta, 5′-GTGTTACTCATAGCGCGTAATATTTGTCTAGGGCC-3′; E1A-gamma, 5′-AATAAGAGGAAGTGAAATCTGAAT AATTTTGTG-3′; and E4, 5′-AGTGACGTATCGTGGGAA AACGGAAG-3′. In addition to synthetic DNA, several experiments used restriction fragments of plasmids to generate longer DNA substrates for footprint and off-rate analyses. These fragments contained E1A sequences from −309 to −145 and E2 sequences from −95 to −31 relative to their mRNA cap sites. The E2 fragment was cloned into the *Sma*I and *Xba*I sites of pGEM2 and then excised as a 100-bp *Eco*RI-to-*Hin*dIII fragment. The E1A fragment was cloned into the *Sma*I and *Xba*I sites of pSP64 and excised as an *Eco*RI-to-*Sal*I fragment.

The 5′ ends of oligonucleotides were ^{32}P labeled in 7-μl reaction mixtures containing 6 μM [γ-^{32}P]ATP (>7,000 Ci/mmol), 0.2 U of T4 polynucleotide kinase, and the standard buffer and salt concentrations used for a T4 DNA polymerase extension reaction (16). Complementary DNA strands were then hybridized by combining them with 10 μl

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of 100 mM NaCl, heating the preparation to 70°C, and slowly cooling it to 25°C. To purify the DNA probe, the mixture was extracted with phenol-chloroform, bound to a 10-μl DEAE-cellulose (DE-52; Whatman, Inc.) column prepared in a pipette tip, washed with 25 ml of 200 mM triethylamine acetate (pH 7), and then eluted in three 600-μl portions of 2 M triethylamine acetate into tubes containing 3 μg of salmon sperm DNA. Triethylamine acetate was removed by lyophilization, and the probe plus carrier DNA was suspended in 200 μl of buffer containing 100 mM NaCl, 10 mM Tris (pH 8), and 1 mM EDTA.

Rather than synthesize both strands of E2-INV, E2+5, and E2+10 DNAs, one strand was prepared and hybridized to a homologous primer DNA that was then extended by using the large fragment of DNA polymerase I. The primer and longer strand were labeled in separate reactions as detailed above.

DNA band shift assays. Band shift assays were performed as described previously (8), with the following modifications. Each reaction contained 3 μl of nuclear extract or column fraction, 10 fmol of ^{32}P-labeled probe DNA, and 1 to 40 μg of DNaseI-digested salmon sperm DNA (30 to 260 bp long). When a DNA with a single E2F site served as substrate for binding, the only salt included in the binding reaction was that supplied in the extract. The low ionic strength led to better resolution for the various E2F complexes during electrophoresis (data not shown). To assay cooperative binding of E2F from infected cells on E2 DNA containing two E2F sites, a binding buffer containing 125 mM NaCl and 1 mM $MgCl_2$ was used. Partially purified E2F from uninfected cells was assayed on E2 DNA in a binding buffer containing 30 mM NaCl and 1 mM $MgCl_2$. After separation of DNA-protein complexes by electrophoresis in a 4% polyacrylamide gel (bisacrylamide-to-acrylamide ratio of 1:20 for relatively smaller synthetic probe DNAs and 1:60 for larger restriction fragments) containing 10 mM Tris hydrochloride (pH 8) and 1 mM EDTA at 4°C, gels were transferred to dampened DEAE paper (DE81; Whatman, Inc.), dried, and then exposed to X-ray film.

Off-rate analysis. The procedure of Fried and Crothers (6) was used to determine the dissociation rates of E2F-DNA complexes. Complexes were generated on a single-site probe DNA by incubating 10 fmol of ^{32}P-labeled E1A site I DNA and 3 μl of nuclear extract in a 10-μl reaction mixture containing 1 μg of salmon sperm DNA, 70 mM NaCl, and 1 mM $MgCl_2$. Complexes were generated on restriction fragments by incubating 10 fmol of either ^{32}P-labeled E1A or E2 double fragments and 3 μl of nuclear extract in 10 μl of reaction mixture containing 40 μg of salmon sperm DNA, 50 mM NaCl, and 1 mM $MgCl_2$. Reaction mixtures were incubated for 1 h at 25°C (a time course experiment demonstrated that the binding reaction had reached equilibrium by this time [data not shown]), and then a 500-fold molar excess of unlabeled E1A site I DNA was added. Equal portions were analyzed by band shift assay as a function of time after addition of the unlabeled competitor DNA.

To ensure that the off-rates followed first-order kinetics, the possible dependence of the rates on the concentration of salmon sperm DNA, ^{32}P-labeled probe DNA, unlabeled E1A site I DNA, and extract was monitored. The rates were independent of all of these parameters (data not shown).

Preparation of extracts and chromatography. Nuclear extracts were prepared according to the protocol of Dignam et al. (5), with minor modifications. Unless otherwise noted, infected-cell extracts were prepared at 24 h after infection with Ad5 at a multiplicity of 20 PFU/ml. In some cases, the extract was dialyzed into buffer containing 50 mM NaCl, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 8), 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, and 20% glycerol. This treatment, however, reduced the level of E2F-a complex observed (Fig. 1B), so the step was generally omitted; the extracts were stored in the 420 mM NaCl extraction buffer. Three protease inhibitors in addition to phenylmethylsulfonyl fluoride were included in extraction buffers (10 mg of leupeptin, 10 mg of soybean trypsin-chymotrypsin inhibitor, and 0.2 U of aprotinin per ml).

Chromatographic procedures were carried out at 4°C. Nuclear extract prepared from either uninfected or Ad5-infected HeLa cells was dialyzed against 100 mM NaCl in buffer A (25 mM HEPES [pH 7.9], 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% [vol/vol] glycerol), clarified by centrifugation at 30,000 × *g* for 10 min, and loaded (10 ml) onto a cation-exchange column (Glass-Pak SP-5PW, 1 by 7.5 cm; Pharmacia/LKB, Inc.) at a flow rate of 0.5 ml/min on a high-performance liquid chromatography (HPLC) system (LKB Instruments, Inc.). After loading, the column was washed with 100 mM NaCl in buffer A (5 ml); it was eluted with a 100 to 400 mM NaCl gradient (30 ml), 400 mM NaCl (5 ml), and a 400 to 1,000 mM NaCl gradient (5 ml), all in buffer A. Fractions (1.5 ml) were collected, commencing with the first gradient. A portion of each fraction was analyzed in a DNA band shift assay. Active fractions were pooled, dialyzed against 100 mM NaCl in buffer A, and loaded onto an anion-exchange column (Glass-Pak DEAE-5PW, 1 by 7.5 cm; Pharmacia/LKB, Inc.), again at a flow rate of 0.5 ml/min. The column was washed with 100 mM NaCl in buffer A (5 ml); it was eluted with a 100 to 320 mM NaCl gradient (30 ml), 320 mM NaCl (5 ml), and a 320 to 1,000 mM NaCl gradient (5 ml), all in buffer A. The two sequential steps achieved a purification of about 200-fold, determined by assaying the amount of total protein needed to occupy 5% of 10 fmol of E1A site I DNA as E2F-specific complexes in a band shift assay containing 1 μg of salmon sperm DNA.

To concentrate E2F activities, active fractions were pooled, dialyzed against 100 mM NaCl in buffer A, loaded onto a pellicular-packed anion-exchange column (MA-7P; Bio-Rad Laboratories), eluted in a small volume of 1 M NaCl in buffer A, and dialyzed against buffer A.

DNase I footprint analysis. Footprint analysis was performed as described by Galas and Schmitz (7) and used E2F activity that was partially purified and concentrated as described above. ^{32}P end labeling of the DNA substrate and

TABLE 1. Sequences of the E2 oligonucleotide and its rearranged variants

Oligonucleotide	Sequence[a]
E2	GATGACGTACTTTCGCGCTTAAATTTGAGAAAGGGCGCGAAACTAGTCCTT
E2-INV	GATGACGTAGCGCGAAAACTTAAATTTGAGAAAGGGCGCGAAACTAGTCCTTA
E2+5	GATGACGTAGTTTCGCGCTTAAATTTGCGTAGAGAAAGGGCGCGAAACTAGTCCTT
E2+10	GATGACGTACTTTCGCGCTTAAATTTTGAGAAATTTGAGAAAGGGCGCGAAACTAGTCCTT

[a] The E2F recognition sites in each oligonucleotide are underlined once; inserted sequences are underlined twice.

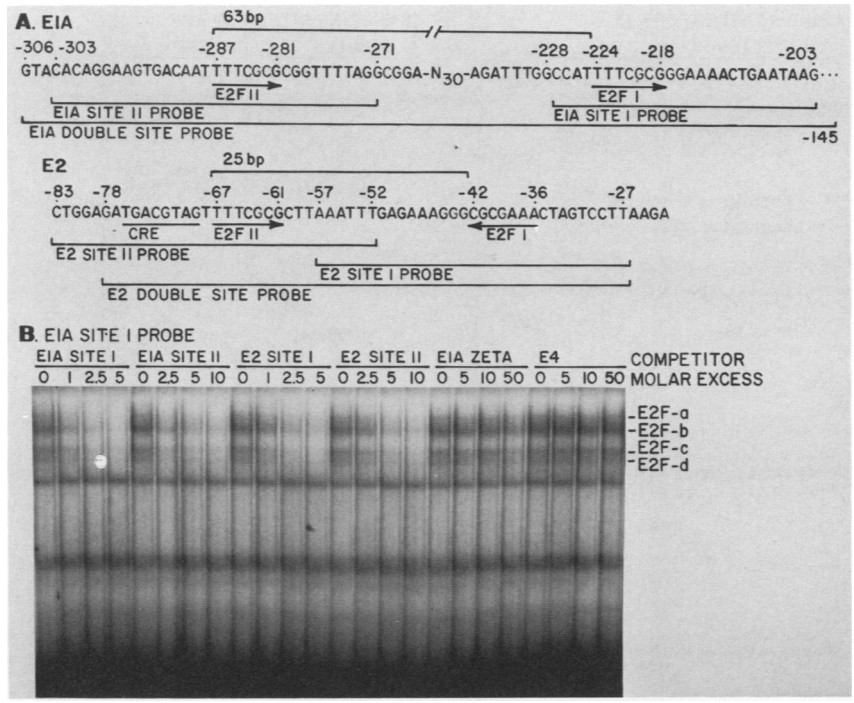

FIG. 1. Sequences of probe DNAs and DNA band shift analysis of uninfected-cell factors that bind DNAs containing a single E2F recognition site. (A) Nucleotide sequences of regions upstream of the E1A and E2 early transcriptional start sites. Numbers above the sequence mark positions relative to the mRNA cap sites at +1. Brackets above the sequence measure the distance between E2F recognition sites that are designated by arrows below the sequence. Brackets below the sequence localize the DNA segments used as probe DNAs throughout this study. (B) DNA band shift analysis of uninfected-cell nuclear extracts, using ^{32}P-labeled E1A site I DNA as a probe. All reaction mixtures contained 1 μg of salmon sperm DNA. Competitions were performed by including in the binding reaction either homologous or heterologous competitor DNAs (identified over groups of four lanes) at the molar excess over probe DNA indicated above each lane. E2F-specific complexes (E2F-a, E2F-b, E2F-c, and E2F-d) are labeled.

binding reactions were carried out as described above, with 100 ng of salmon sperm DNA per reaction.

UV crosslinking. After binding of E2F to ^{32}P-labeled E1A site I DNA in 100 ng of salmon sperm DNA as described above, the reaction mixtures (10 μl) were pipetted onto parafilm and irradiated for 4 min at a distance of 4.5 cm from a UV lamp (300-nm filter; Fotodyne). DNA was then digested for 30 min at 37°C with 2 U of micrococcal nuclease (Sigma Chemical Co.) in buffer containing 10 mM $CaCl_2$. Photoadducts were analyzed by electrophoresis in an 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). After electrophoresis, the gel was dried onto filter paper and exposed to film, using an intensifying screen.

Densitometry. The intensities of bands were quantified by densitometry of autoradiographs prepared by using film preflashed to an optical density of 0.1 and exposed without the use of an intensifying screen. Exposures of optical density up to 1.0 were then quantified by using a densitometer (Bio-Rad).

RESULTS

E2F activities from uninfected HeLa cells form multiple complexes with DNAs. We initially observed E2F activity in an extract of uninfected HeLa cell nuclei by using the band shift assay of Fried and Crothers (6). Since appreciable levels of E2F activity have not been reported in extracts from uninfected cells, we sought to confirm that this activity was E2F. The Ad5 chromosome contains four known E2F binding sites (13, 14) (Fig. 1A). We tested the ability of the putative E2F activity to complex with four synthetic DNA probes, each containing one of the binding sites. These double-stranded oligonucleotides were designated E1A site I, E1A site II, E2 site I, and E2 site II (Fig. 1A). To minimize the chance that oligonucleotides would bind additional activities besides E2F, the size of each DNA was limited to about 30 bp.

Several complexes were observed when the E1A site I oligonucleotide was used as a labeled probe in a band shift assay (Fig. 1B, leftmost lane). To determine which of these complexes resulted from interaction at the E2F site, band shift assays were performed, using unlabeled oligonucleotides as competitors. Each DNA containing an E2F site (TTTCGCG) competed for the label in the four slowly migrating complexes (E2F-a, E2F-b, E2F-c, and E2F-d; Fig. 1B). Two oligonucleotides with sequences related to the E2F site were also tested for the ability to compete for E2F binding. The E1A-zeta DNA (containing the sequence AT TCGCG) and the E4 DNA (containing the sequence TTTC CCA) exhibited little or no ability to compete for E2F binding (Fig. 1B).

From these data, it is clear that four complexes were formed with extracts from uninfected cells with specific sequences of E1A site I DNA. The most extensive similarity between the oligonucleotides that effectively competed was the sequence TTTCGCG, the E2F site. All DNAs containing an E2F site competed with approximately the same efficiency, which, apart from implicating the E2F site as the locus of binding, showed that the E2F-like activity had approximately the same affinity for all four oligonucleotides. By the same reasoning, the E2F-a, E2F-b, E2F-c, and E2F-d

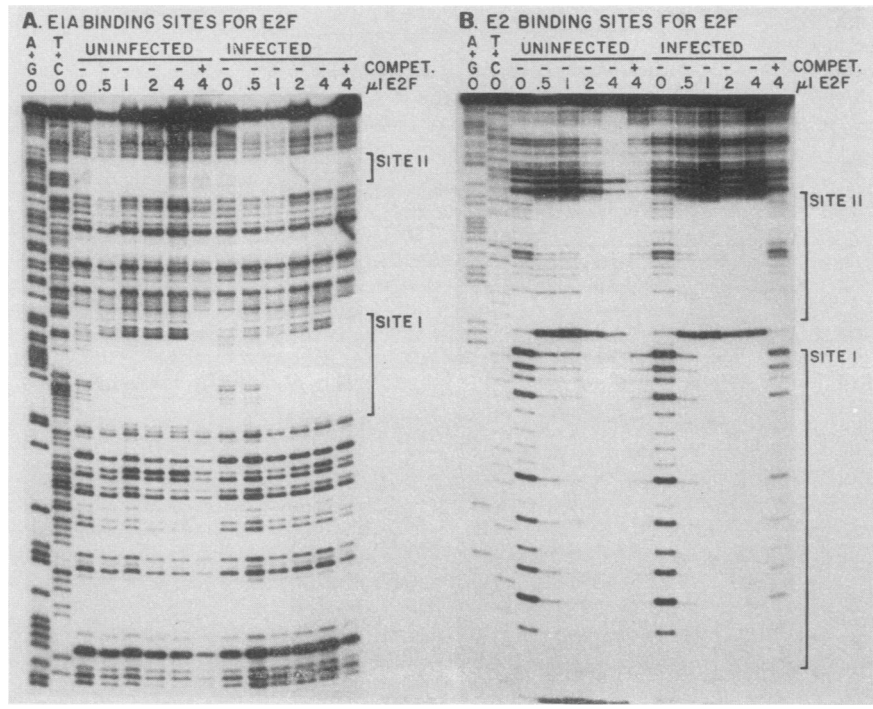

FIG. 2. DNase I footprint analysis of partially purified E2F from uninfected and infected HeLa cells on Ad5 DNA fragments. Assays were performed by using either E1A (A) or E2 (B) DNA restriction fragments as the end-labeled probe DNA. Partially purified and concentrated E2F activity was prepared as described in Materials and Methods; the amount of E2F activity added to each reaction is indicated above each lane. Reactions containing competitor DNA included a 100-fold molar excess of unlabeled E1A site I DNA. Protected regions are designated by brackets; the sequences labeled site I and site II correspond to the E2F sites shown in Fig. 1A. Products of sequencing reactions (A+G and T+C) are included as markers.

complexes formed with E1A site I DNA all resulted from interaction at the E2F site, since each of the four oligonucleotides competed for binding of all four complexes. Furthermore, the four specific bands decreased in intensity over the same range of competitor DNA concentration, indicating that the four complexes had the same dependence on DNA concentration.

Of the E2F-specific complexes, the faint E2F-a complex varied in intensity with different extract preparations (discussed in Materials and Methods). In addition to the specific complexes, there were several nonspecific complexes that migrated faster than the specific species.

To further demonstrate that all four E2F sites were bound by the same activity, we compared the pattern of shifted bands obtained using the four different single-site oligonucleotides as ^{32}P-labeled probes (data not shown). All of the DNAs produced a series of four complexes corresponding to the E2F-a, E2F-b, E2F-c, and E2F-d complexes identified above.

We conclude that there are activities in uninfected HeLa cells that bind specifically to E2F sites. They form several complexes with the E2F recognition site. We have termed these complexes E2F-a, E2F-b, E2F-c, and E2F-d.

Uninfected- and infected-cell E2F activities protect identical sequences in a footprint assay. The E2F activity from adenovirus-infected cells has been shown to protect the sequence TTTCGCG in DNase I footprint assays (13). The activity from uninfected cells was tested to determine whether it generated the same pattern of protection. E2F activities were partially purified and concentrated (see Materials and Methods) from uninfected and Ad5-infected HeLa cells and assayed, using restriction fragments containing the E2F sites upstream of either E1A or E2.

As increasing amounts of partially purified E2F were added to an end-labeled E1A DNA, two regions (sequence positions 268 to 284 [site I] and 205 to 218 [site II]) were protected from DNase I digestion (Fig. 2A). The sequence at the center of each protected region was TTTCGCG, the E2F binding site. E2F from infected and uninfected cells generated the same pattern of protection. Excess unlabeled E1A site I DNA interfered with the protection (Fig. 2A), indicating that the footprint analysis and band shift assays performed with the single-site oligonucleotide detected the same activity.

The analysis was repeated by using end-labeled E2 DNA (Fig. 2B). There were two protected regions at −35 to −53 (site I) and −57 to −73 (site II) relative to the E2 early transcriptional start site. In addition, there was a DNase I-hypersensitive site at −54. E2F prepared from uninfected or infected cells generated the same pattern of protection.

Ad5 infection alters the pattern of E2F complexes observed in a band shift assay. The E2F-specific complexes detected by band shift assay were monitored for changes after Ad5 infection of HeLa cells. Nuclear extracts were prepared at 0, 6, and 24 h after infection and assayed, using the E1A site I oligonucleotide as a ^{32}P-labeled probe (Fig. 3A). At 6 h after infection, a new complex (designated E2F-e) that increased in amount by 24 h was detected. This new complex formed at the E2F binding site, since its production could be prevented by competition with each of the four oligonucleotides containing E2F recognition sites but not by heterologous DNAs that lacked E2F binding sites, and each of the four E2F-specific oligonucleotides produced the same new complex when used as probes in band shift assays (data not shown). At 24 h after infection, the E2F-c complex was undetectable and the E2F-e complex was the predominant

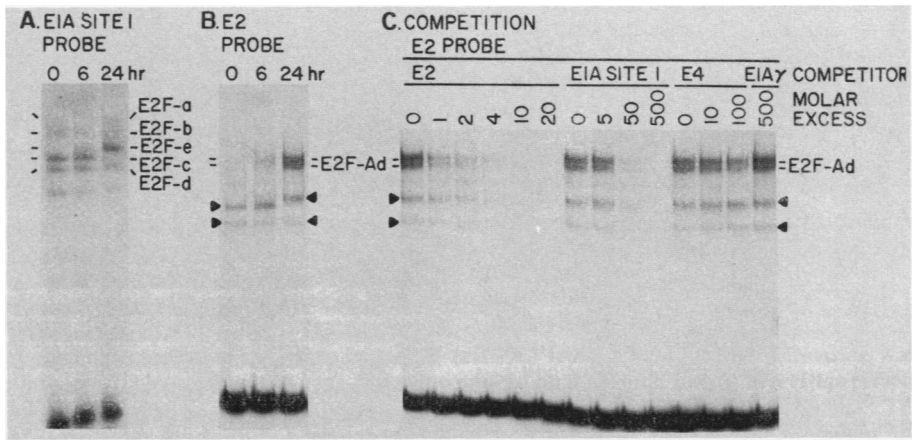

FIG. 3. DNA band shift analysis of E2F activities in nuclear extracts prepared at various times after infection with Ad5. (A) Assays performed with 1 μg of salmon sperm DNA and 10 fmol of ^{32}P-labeled E1A site I DNA as a probe, with extracts prepared at 0, 6, or 24 h after infection with Ad5. E2F-specific complexes (E2F-a, E2F-b, E2F-c, E2F-d, and E2F-e) are labeled. (B) Assays performed with 40 μg of salmon sperm DNA and 10 fmol of ^{32}P-labeled E2 restriction fragment as a probe, with the same extracts as in panel A. The infection-specific double-site occupancy complexes (E2F-Ad) are labeled. Single-site occupancy complexes that are E2F specific are marked by arrowheads. (C) Competition analyses using a ^{32}P-labeled E2 restriction fragment as a probe and homologous or heterologous unlabeled competitor DNAs (identified above groups of lanes) at the molar excess over probe DNA indicated above each lane. Labels are as in panel B.

form, suggesting the possibility that the activity giving rise to the E2F-c complex had been converted to an activity that generated the E2F-e complex. No infection-induced increase in the overall level of E2F binding activity was observed in the experiment shown in Fig. 3A. Although a three- to fivefold increase in binding to the E1A site I DNA was observed with extracts prepared late after adenovirus infection in some experiments (data not shown), we did not see the dramatic increase reported by Kovesdi et al. (13).

Since we failed to see a substantial increase in E2F binding activity during adenovirus infection, the experiment was repeated with a DNA probe similar to the one used by Kovesdi et al. (13). Instead of a single E2F site, a DNA restriction fragment from upstream of the E2 cap site that spanned two E2F sites was used. Using this probe, we observed induction of a pair of new complexes, designated E2F-Ad, that represented a substantial increase in binding activity (Fig. 3B). The E2F-Ad doublet was very likely the same as that identified by Kovesdi et al. (13). The induction of the E2F-Ad doublet paralleled the appearance of the E2F-e complex detected with a single-site probe (compare Fig. 3A and B). In addition to E2F-Ad, two faster-migrating complexes were detected (arrowheads in Fig. 3B) with the double-site probe. The upper of these complexes detected in uninfected cells (Fig. 3B, 0-h lane) appeared to be replaced by a slower-migrating species as the infection proceeded (Fig. 3B, 24-h lane). This paralleled the apparent replacement of E2F-c with the E2F-e complex on the E1A site I probe (Fig. 3A) and supports assignment these complexes as the E2F-c, E2F-d, and E2F-e complexes formed on the double-site-containing DNA. In fact, when a 53-bp E2 double-site oligonucleotide was used as a probe, these complexes comigrated with E2F-c, E2F-d, and E2F-e formed on E1A site I, verifying their identities (data not shown).

To examine the nature of the complexes observed on the E2 DNA restriction fragment, we used an unlabeled E2 fragment, E1A site I DNA, and two nonspecific DNAs as competitors for binding to the labeled E2 DNA fragment (Fig. 3C). Competition with the unlabeled E2 fragment demonstrated that the four predominant complexes (E2F-Ad and the bands marked by arrowheads) resulted from interaction at specific sequences. Competition with E1A site I DNA showed that the complexes were E2F specific, and nonspecific DNA had little effect.

Thus, the band shift assay detected three changes in E2F activity after infection of cells by adenovirus. Using a DNA probe containing a single E2F site, we detected an infection-specific E2F complex, E2F-e, but very little increase in E2F binding activity. Using a DNA probe containing two E2F sites from the E2 control region, we detected a large increase in binding activity and a pair of infection-specific complexes, E2F-Ad.

E2F-Ad and E2F-e copurify through two high-resolution chromatographic separations. To probe the relationship between the two infection-specific E2F complexes (E2F-e and E2F-Ad), a nuclear extract from Ad5-infected HeLa cells was chromatographed through two successive HPLC ion-exchange columns. Fractions were assayed by DNA band shift analysis, using both the E1A site I and E2 double-site oligonucleotides (Fig. 1A). The activities producing the E2F-d and E2F-e complexes on E1A site I DNA copurified with the activity generating E2F-Ad on E2 double-site DNA during cation-exchange chromatography (SP-5PW) but separated from the activities giving rise to E2F-a and E2F-b complexes (data not shown). The fractions with peak infection-specific activity (E2F-e and E2F-Ad) were pooled, dialyzed, and applied to an anion-exchange column (DEAE-5PW). An interesting relationship between the E2F-e and E2F-Ad complexes is evident in the elution profile (Fig. 4). During this chromatographic step, the activities that produced the E2F-d and E2F-e complexes were separated such that fraction 10 contained the activity that generated E2F-e, with little of the other E2F activities (Fig. 4A). The same fraction contained activity generating the upper complex of the E2F-Ad doublet (Fig. 4B).

Each observed complex was the product of a chromatographically separable E2F activity. Of these resolved E2F activities, only the E2F-e activity copurified with the activity that generated the upper E2F-Ad complex. Therefore it is likely that this upper E2F-Ad complex formed from E2F-e activity. The composition of the lower E2F-Ad complex is more difficult to understand since its peak activity was in

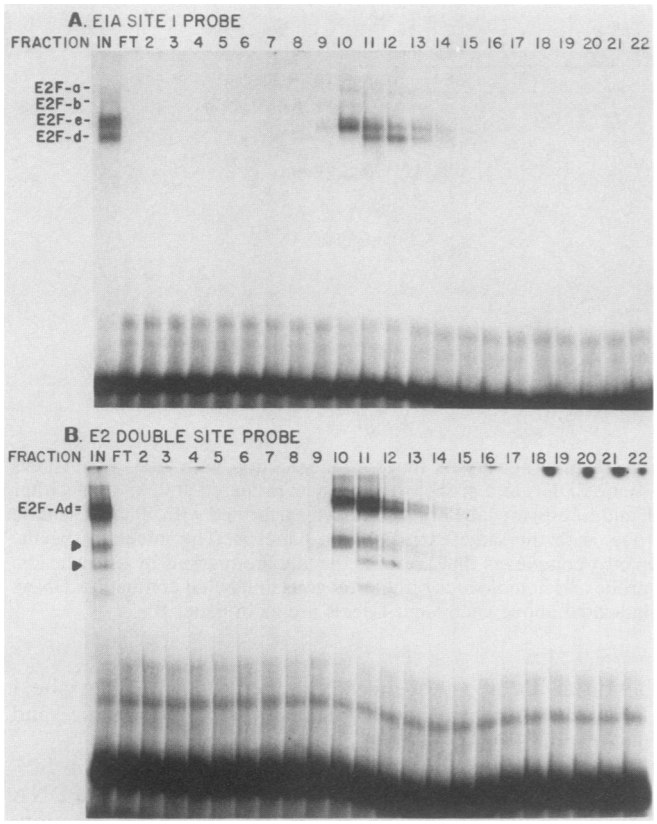

FIG. 4. DNA band shift analysis of E2F activities in fractionated nuclear extracts prepared from Ad5-infected HeLa cells, using single-site versus double-site probe DNAs. Extract prepared at 24 h postinfection was first separated on an SP-5PW column, fractions with E2F activity were identified by band shift assay, and material from the active fractions was further analyzed by chromatography on a DEAE-5PW column, analysis of which is shown. (A) Band shift assay using 1 μg of salmon sperm DNA and ^{32}P-labeled E1A site I DNA as a probe. E2F-specific complexes (E2F-a, E2F-b, E2F-c, E2F-d, and E2F-e) are labeled. (B) Band-shift assay using 5 μg of salmon sperm DNA and ^{32}P-labeled E2 DNA as a probe. The infection-specific doublet (E2F-Ad) is labeled. Single-site occupancy complexes that are E2F specific are marked by arrowheads. IN, Input; FT, flowthrough.

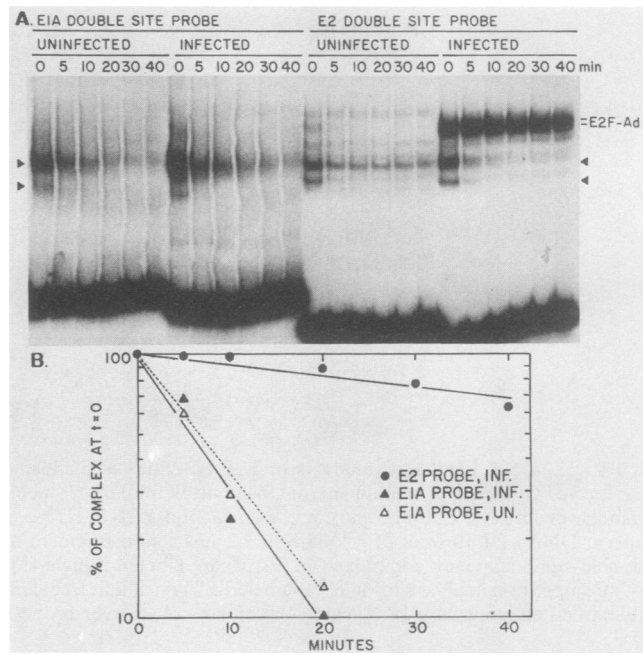

FIG. 5. Off-rate analysis of E2F complexes formed on DNA restriction fragments containing two E2F recognition sites. (A) Band shift analysis of the off-rate experiment. Complexes were formed in 40 μg of salmon sperm DNA on either ^{32}P-labeled E1A or ^{32}P-labeled E2 DNA, using nuclear extracts of uninfected or Ad5-infected cells, a 500-fold molar excess of unlabeled E1A site I DNA was added, and samples were taken for electrophoretic analysis at the times after addition of competitor indicated above the lanes. Each sample was loaded on a running gel as soon as it was taken. As a result, samples were subjected to electrophoresis for somewhat different time periods, leading to small differences in the apparent mobilities of complexes and free DNA. (B) Quantitative plot of off-rate data. The intensities of bands representing E2F complexes were quantified by densitometry; the percentage of complex remaining was calculated and plotted as a function of time after addition of competitor. Shown are data for E2F-specific complex formed on an E2 probe with infected-cell extract and on an E1A probe with infected-cell and uninfected-cell extracts.

fraction 11, between the maxima of E2F-e and E2F-d activities. This result suggests that it may be dependent on both activities for its formation and that the complex may contain some of each type of activity. The data are insufficient to determine whether E2F-d can participate in the E2F-Ad complex without E2F-e.

Infected-cell extracts produce long-lived complexes on E2 but not E1A double-site DNAs. To further compare the infected- and uninfected-cell extracts, we performed off-rate analysis of E2F complexes formed in the unfractionated extracts. Since we have observed radically different binding behaviors in comparisons of a DNA containing two E2F sites and DNA with any single E2F site, two double-site DNA probes from the E1A and E2 control regions were tested by this analysis. ^{32}P-labeled double-site DNAs (10 fmol) plus 40 μg of salmon sperm DNA were incubated in extracts of uninfected or Ad5-infected HeLa cell nuclei until E2F binding reached equilibrium. Then a 1,000-fold molar excess of unlabeled E1A site I DNA (170 ng) was added, and portions were removed at various times for the next 40 min and analyzed by the band shift assay (Fig. 5A). The complexes were quantified by densitometry and plotted as a function of time to determine the decay rates of the E2F complexes (Fig. 5B).

The double-site E1A DNA probe generated two major complexes in either uninfected- or infected-cell extract (arrowheads in Fig. 5A). Since the E1A site I DNA competed for the formation of both complexes, these were probably E2F complexes. In contrast, a nonspecific oligonucleotide had no effect (data not shown). The half-lives of the two complexes were not affected by infection. The more abundant complex exhibited a half-life of about 15 min (Fig. 5B). The less abundant complex had a half-life of less than 1 min (Fig. 5A).

Using the E2 double-site restriction fragment, we observed that a number of complexes formed in both infected and uninfected extracts, with the E2F-Ad complexes forming only in the infected extract (Fig. 5A). The E2F-Ad complex exhibited a half-life of about 1 h, considerably longer than the half-lives of complexes that formed on the E1A double-site DNA (Fig. 5). The other complexes formed between E2 DNA and E2F activities in either uninfected- or infected-cell extract (E2F-c, and E2F-d, and E2F-e; arrowheads in Fig. 5A) had short half-lives, similar to the com-

plexes formed on the E1A double-site DNA. For both types of extract, there were several complexes formed on the E2 DNA that were not competed against by the E1A site I DNA. These complexes were competed against by an oligonucleotide containing a cyclic AMP response element (data not shown), indicating that the complexes formed on this element adjacent to E2F site II (Fig. 1A). A complex was formed on the E2 DNA in uninfected extracts that appeared to comigrate with the E2F-Ad complex (Fig. 5A). The activity that formed this complex did not copurify with the E2F species that generated the E2F-Ad complex, however (data not shown).

To complete this analysis, we also used E1A site I as a probe DNA. The four E2F activities observed on the E1A site I DNA separated into two classes with respect to half-life, similar to the results obtained with the E1A double-site probe. The half-lives of the E2F-a and E2F-b complexes were about 10 times the half-lives of E2F-c, E2F-d, and E2F-e (data not shown).

These off-rate analyses demonstrate a major difference between the DNA fragments containing pairs of E2F sites from the control regions of E1A and E2 in their interactions with infected cell E2F. A relatively long-lived E2F complex (E2F-Ad) formed solely on the E2 double-site DNA when incubated in an infected cell extract. In contrast, only relatively short-lived complexes formed on E1A double-site DNA in both infected and uninfected extracts. This infection-specific difference between E2 and E1A was not seen with single E2F sites from either source (data not shown). Therefore, the long lifetime of the E2F-Ad complex is not explained by an infection-generated increase in the bound lifetime of E2F on single E2F sites. This conclusion suggests that the different responses of the E1A and E2 DNA fragments were a result of either the arrangement of E2F sites or the sequences surrounding the binding sites.

Non-first-order binding of infected-cell E2F activity to the double-site E2 DNA. Apart from differences in sequences surrounding the E2F sites in the E1A and E2 control regions, the relative orientation of each pair of sites and the distance separating each pair were different (Fig. 1A). The two E2F sites were separated by 63 bp (center to center) forming a direct repeat in E1A DNA and by 25 bp forming an inverted repeat in E2 DNA. Thus, the contrasting behaviors of complexes formed on E2 compared with E1A double-site DNAs during the off-rate analysis could have resulted from the relative spacing and orientation of the E2F sites, suggesting an interaction between pairs of sites. In other words, the binding of an infection-specific form of E2F to E2 DNA might be cooperative.

The nature of the E2F-Ad complex detected in the band shift assay using the E2 double-site probe DNA was examined by combined band shift-footprint analysis (Fig. 6) (2). DNA-protein complexes were treated with DNase I before separation by electrophoresis on a native gel. The E2F-Ad doublet was excised from the gel, and the cleavage pattern of the DNA was analyzed by electrophoresis on a denaturing gel. E2F sites I and II were both protected, and a hypersensitive site at +54 was evident. Simultaneous occupancy of the two sites in the E2F-Ad complex is one requirement of a complex formed by cooperative binding.

Simultaneous occupancy of two E2F sites in the E2F-Ad complex could result from either a single factor binding to both sites or two factors, each binding one site. The latter situation is consistent with cooperative binding in two steps, and the former is consistent with a dimer of E2F forming and then binding to the DNA or an interaction of a single protein

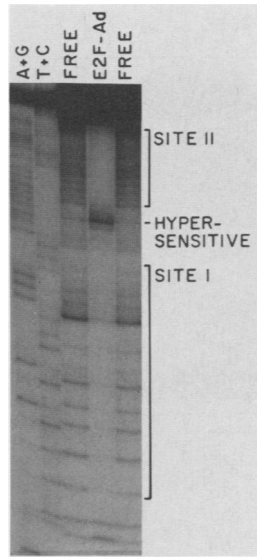

FIG. 6. DNase I footprint analysis of the E2F-Ad complex. Partially purified E2F from infected cells and end-labeled E2 DNA were used to prepare E2F-Ad complex. After binding, the reaction components were treated with DNase I. E2F-Ad complex and free, uncomplexed DNA were isolated and then analyzed by electrophoresis under denaturing conditions. Protected regions (sites I and II) as well as a hypersensitive site are labeled. Products of sequencing reactions (A+G and T+C) are included as markers.

with two binding sites. To distinguish between these possibilities, we performed binding assays in which the ^{32}P-labeled E2 DNA concentration was kept constant and the amount of infected cell extract was progressively increased (Fig. 7A). The total protein concentration in each reaction mixture was held constant by diluting the extract in buffer containing 1 mg of bovine serum albumin. The various E2F complexes were quantified and plotted as a function of extract concentration (Fig. 7B). The E2F-Ad complex exhibited a nonlinear response to increasing protein concentration, producing a sigmoidal dose-response curve. This result is consistent with there being more than one factor involved in the E2F-Ad complex.

In contrast to the response of the E2F-Ad complex, the E2F-e complex increased linearly with increasing extract concentration at a low extract concentration (Fig. 7). This result is consistent with a simple interaction of a single E2F molecule with an E2 DNA molecule.

Apart from the shapes of the curves representing the E2F-Ad and E2F-e complexes, the relative amounts of the complexes were also suggestive of cooperative binding. The E2F-e curve reached a plateau with about 2% of the E2 DNA bound. In contrast, the amount of the E2F-Ad complex was less than the amount of the E2F-e complex at a very low extract concentration and then far exceeded the amount of E2F-e, reaching a plateau with about 60% of the E2 DNA bound. This result shows that the E2F-Ad complex was favored over the E2F-e complex as the protein concentration increased, a characteristic of cooperative binding.

For comparison, this analysis was repeated with E2F from uninfected cells. Since we could not find evidence of doubly occupied complex formation by using an unfractionated extract, partially purified and concentrated E2F-c and E2F-d activity was used (see Materials and Methods). The band shift assay of this titration is displayed in Fig. 8A. The

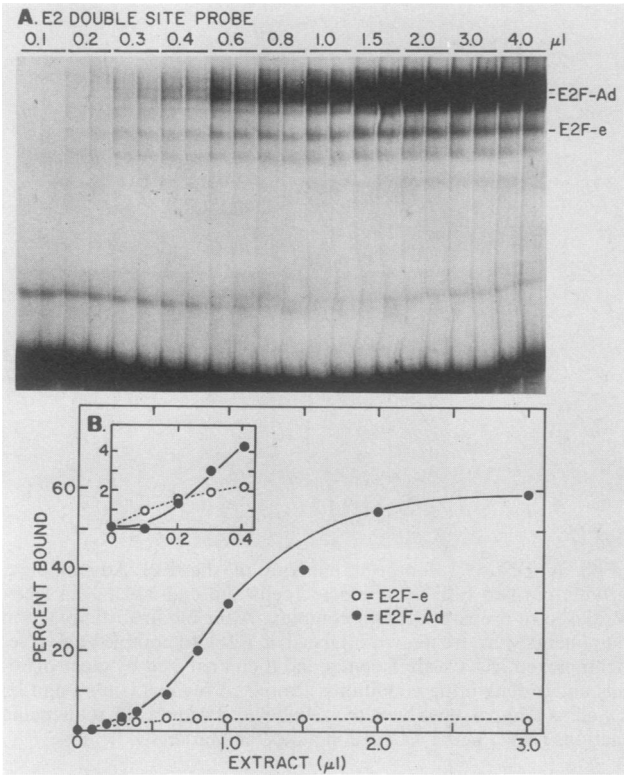

FIG. 7. Titration of E2F activity from infected cells on the E2 double-site DNA probe. (A) Band shift analysis of the titration. Increasing amounts (indicated above duplicate lanes) of infected-cell nuclear extract were mixed with a constant amount of ^{32}P-labeled E2 probe DNA and 5 μg of salmon sperm DNA, and the resulting complexes were analyzed by electrophoresis. Complexes containing E2F at only one of its two recognition sites on E2 DNA and doubly occupied complexes are labeled E2F-e and E2F-Ad, respectively. (B) Quantitative plot of titration data. The densities of the free DNA signal as well as of singly and doubly occupied complexes were quantified by densitometry. The percentage of probe DNA bound in each type of complex was calculated and plotted as a function of extract concentration.

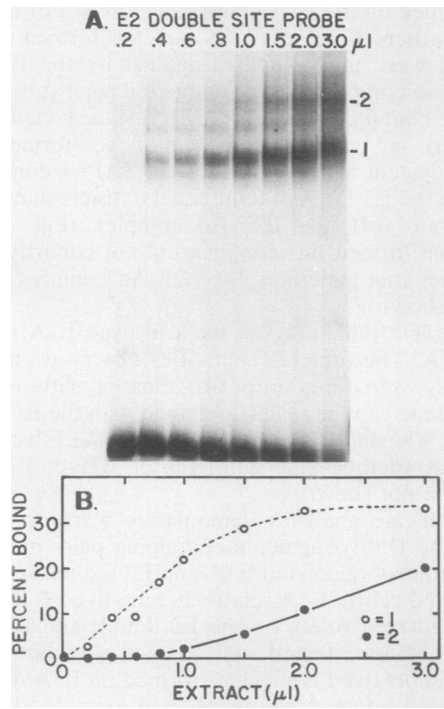

FIG. 8. Titration of E2F activity from uninfected cells on the E2 double-site DNA probe. (A) Band shift analysis of the titration. Increasing amounts (indicated above the lanes) of uninfected-cell nuclear extract were mixed with a constant amount of ^{32}P-labeled E2 probe DNA and 1 μg of salmon sperm DNA, and the resulting complexes were analyzed by electrophoresis. Complexes containing E2F at only one of its two recognition sites on E2 DNA and doubly occupied complexes are labeled 1 and 2, respectively. (B) Quantitative plot of titration data. The densities of the free DNA signal as well as of singly and doubly occupied complexes were quantified by densitometry. The percentage of probe DNA bound in each type of complex was calculated and plotted as a function of extract concentration.

complexes labeled 1 and 2 were E2F specific, since they could be eliminated by inclusion of excess unlabeled E1A site I DNA. The pair of complexes labeled 2 comigrated with the E2F-Ad complexes (data not shown) and, like the E2F-Ad complexes, they had a nonlinear dependence on extract concentration (Fig. 8B). This finding indicates that these complexes resulted from interaction of more than one factor with the E2 DNA. In all probability, these are complexes of two molecules of E2F per E2 DNA. The complexes labeled 1 had a linear dependence on extract concentration at low protein concentrations. This and the comigration of these complexes with the E2F-c complex formed on single-site DNA (data not shown) indicate that these complexes contained one E2F molecule per E2 DNA. We also examined this complex by the combined footprint-band shift assay. The resulting footprint showed partial protection over both E2F sites, a result consistent with the complex being a mixture of the E2F activity bound at either site I or site II (data not shown).

In sum, the single-site complex predominated at all concentrations of uninfected-cell extract tested. The doubly occupied complex (labeled 2) began to approach the level of the singly occupied complex when the latter included about 30% of the E2 DNA. These results are consistent with very little cooperativity, if any, between the two sites. Therefore, E2F from uninfected-cell extracts behaved in a manner different from that of E2F from infected-cell extracts.

Relative orientation and spacing of recognition sites influence binding of E2F from infected cells. Cooperative binding could depend on the orientation and spacing of sites in the substrate DNA. To test this possibility, several variant oligonucleotides were prepared in which the E2F binding sites in the double-site E2 DNA were rearranged (Fig. 9A). The orientation of site II was reversed without altering the spacing between the two sites on the E2-INV oligonucleotide. The spacing between sites I and II was increased without altering the relative orientations of these sites on the E2+5 and E2+10 oligonucleotides. The E2+5 alteration moved site II to the opposite face of the helix relative to site I as compared with its normal position, whereas E2+10 restored the sites to their normal relative helical faces.

Partially purified E2F from infected cells generated substantial quantities of the E2F-Ad double-site occupancy complexes on the E2 and E2+10 DNAs and very little of the complex on the E2-INV and E2+5 DNAs (Fig. 9B). The clear differences between E2 DNA and the rearranged DNAs demonstrated an orientation and spacing dependence of infection-specific E2F binding. The sites had to be arranged as an inverted repeat, since E2-INV DNA failed to

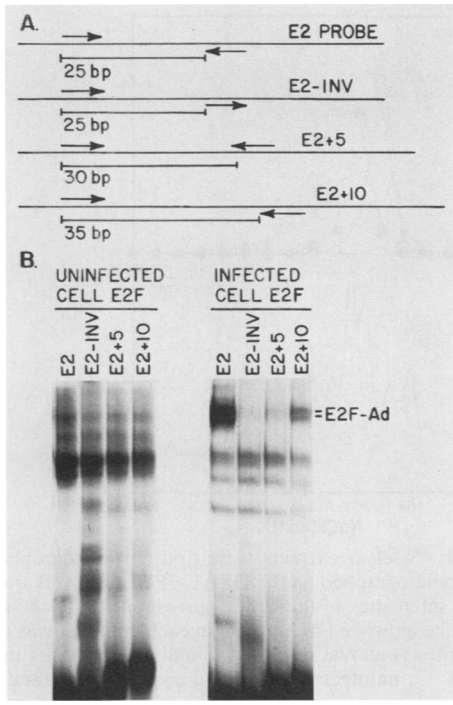

FIG. 9. Assay for the dependence of E2F binding on the orientation and spacing of E2F sites in E2 DNA. (A) Diagram of the arrangement of E2F sites in E2 DNA and rearranged variant DNAs. Arrows indicate orientations of sites; the bar under each schematic indicates the spacing between sites. (B) Band shift assay of partially purified E2F from uninfected or Ad5-infected cells on E2 DNA and its rearranged variants. Each binding reaction contained 5 μg of salmon sperm DNA. Bands representing the E2F-Ad complex are labeled.

efficiently generate the E2F-Ad complex. The sites had to be related in terms of the face of the DNA helix they occupied, since E2 and E2+10 produced more of the E2F-Ad complexes than did E2+5. These spatial requirements are consistent with cooperative binding by E2F at the pair of sites.

When partially purified E2F from uninfected cells was assayed, the E2 DNA and all three variant DNAs generated the same set of complexes (Fig. 9B). This result makes sense if the uninfected-cell E2F bound independently to the two E2F recognition sites. All four DNAs generated complexes that comigrated with the E2F-Ad complexes, demonstrating that the altered sites were still functioning for binding. The apparent formation of more double-site occupancy complex on E2-INV and E2+5 DNAs by E2F from uninfected cells than by E2F from infected cells was simply due to the fact that more activity from uninfected cells was added to the assays. When a large amount of infection-specific E2F was added, double-site complexes formed on all DNAs. These results also suggest that only the E2F sites are involved in E2F-Ad complex formation and that other DNA-binding sites are not involved.

E2+10 DNA clearly generated E2F-Ad complex more efficiently than did E2-INV or E2+5 DNAs. To better analyze the degree to which cooperative binding occurred on the E2+10 DNA, partially purified E2F from infected cells was titrated on E2, E2-INV, and E2+10 DNAs and assayed by band shift assay (data not shown). An estimate of relative E2F binding cooperativity on the different DNAs was obtained by comparison of the relative amount of single-site occupancy complex to double-site occupancy complex (E2F-Ad). E2 DNA generated much more double-site than single-site occupancy complex, E2-INV DNA gave the opposite result, and E2+10 DNA produced on intermediate ratio of the two complexes. The additional 10 bp between the E2F recognition sites in E2+10 DNA reduced but did not completely prevent cooperativity.

E2F activities from uninfected and Ad5-infected HeLa cells are related. Although the activities that formed E2F complexes in infected- and uninfected-cell extracts interacted with the same sequences, their dissimilar binding properties suggested that they could be unrelated factors. Perhaps the synthesis of a different polypeptide that recognizes the same DNA sequence is induced by adenovirus infection. Alternatively, the infected- and uninfected-cell E2F activities might be the same factor differing by a posttranslational modification. Two experiments were performed to assess the relationship of E2F activities derived from infected and uninfected cells.

First, the chromatographic behaviors of E2F activity from the two sources were compared. Uninfected and infected nuclear extracts were fractionated sequentially, first on an HPLC cation-exchange matrix (SP-5PW) and next on an HPLC anion-exchange matrix (DEAE-SPW). The amounts of the E2F-c, E2F-d, and E2F-e activities were quantified and plotted as a function of the ionic strength of each fraction in order to align the different chromatographic separations (Fig. 10). The E2F-c and E2F-e activities copurified. In addition, E2F-d activities from both sources copurified, as might be expected, whereas the E2F-d activities were resolved from E2F-c and E2F-e. Thus, the infection-specific form (E2F-e) and a normal cellular form (E2F-c) were sufficiently related to copurify through two sequential chromatographic separations.

The relationship between E2F-c and E2F-e was further probed by cross-linking the E2F molecules to labeled DNA. This technique has been used to transfer label from DNA to a variety of DNA-binding proteins, including the *lac* repressor (15), and, more recently, a factor that binds to the adenovirus major late transcriptional control region (3, 20). ^{32}P-labeled E1A site I oligonucleotide was incubated in nuclear extracts from uninfected or infected cells and irradiated with UV light, and the cross-linked material was analyzed by electrophoresis. A pronounced band migrating at 64 kDa was generated by this procedure (Fig. 11). The intensity of the 64-kDa band increased linearly with increasing time of exposure to UV light (Fig. 11B), consistent with a simple interaction between the activated DNA and a closely associated protein. The cross-linked band could be detected in either uninfected or infected nuclear extracts or in partially purified preparations of E2F activity (Fig. 11A). Cross-linking to the 64-kDa band could be inhibited by addition of unlabeled E1A site I DNA to either uninfected- or infected-cell reactions, and cross-linking of the infected-cell activity was also prevented by adding E1A site II, E2 site I, or E2 site II DNA as competitor (Fig. 11A). These results show that both infected- and uninfected-cell extracts contained an activity of 64 kDa that could be cross-linked to an E2F site.

These cross-linking assays showed that one of the E2F activities produced a 64-kDa photoproduct but did not identify which E2F activity was being cross-linked. All of the E2F preparations tested contained the E2F-d activity. Therefore, the 64-kDa photoadduct could simply reflect cross-linking of only E2F-d activity. To determine whether the infection-specific form, E2F-e, also generated a 64-kDa product, the fractions from the DEAE-5PW chromatography

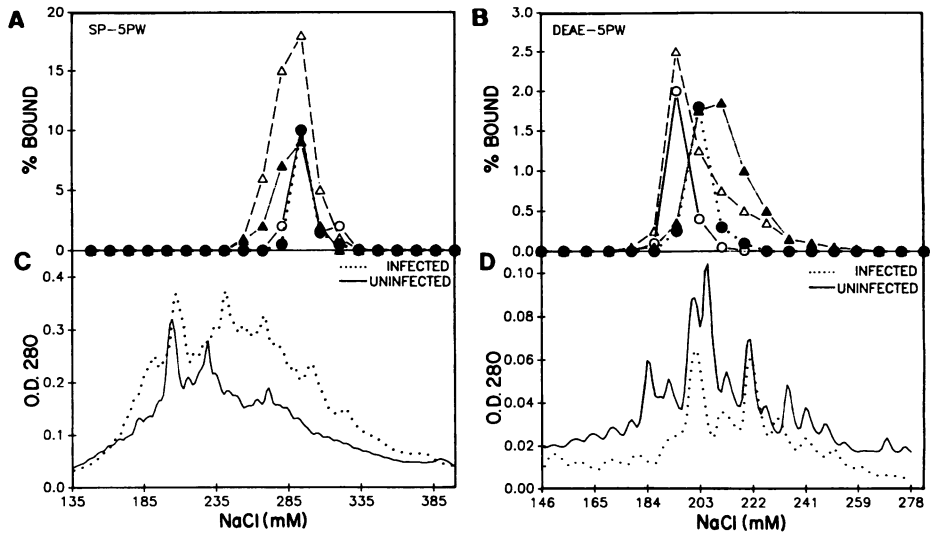

FIG. 10. Copurification of E2F activities from uninfected and Ad5-infected cells. Nuclear extracts were first chromatographed on the SP-5PW matrix (A and C), and then fractions with E2F activity were pooled and chromatographed on the DEAE-5PW matrix (B and D). E2F activities were assayed by band shift assay, using E1A site I DNA as a probe. The intensities of the bands corresponding to the free DNA signal and E2F-specific complexes were quantified by densitometry, and the percentage of probe DNA bound in each complex was calculated and plotted (A and B). Total optical density at 280 nm (O.D. 280) for elution profiles is shown in panels C and D. Fractions in different chromatographic runs were aligned by ionic strength. Symbols for panels A and B: ○, uninfected-cell E2F-c complex; ●, uninfected-cell E2F-d complex; △, infected-cell E2F-e complex; ▲, infected-cell E2F-d complex.

of infected extract were examined by photo-cross-linking. The activity generating the 64-kDa band in the cross-linking assay (Fig. 11C) and the activity generating the infection-specific E2F complexes (E2F-e and E2F-Ad; Fig. 4) coeluted from the DEAE-5PW column. The fact that fraction 10 contained E2F-e activity relatively free of the other E2F activities strongly suggests that the infection-specific form was a 64-kDa protein.

The copurification of the infection-derived form of E2F with an uninfected-cell form suggests that the two factors have a very high degree of similarity. This and the finding that the infection-specific form and uninfected-cell forms were both approximately 64 kDa is strong evidence that they differ by posttranslational modification.

DISCUSSION

The main conclusion of this work is that E2F undergoes a change in adenovirus-infected cells that alters its binding properties. E2F from infected cells binds in a cooperative fashion to pairs of E2F sites, whereas E2F from uninfected cells binds to pairs of sites with little cooperativity. Cooperative binding depends on the relative arrangement of the two E2F sites. The naturally occurring arrangement upstream of the E2 early start site allows cooperative binding, whereas the arrangement of sites upstream of the E1A start site does not. These regions differ in both the relative orientation and spacing of E2F binding sites (Fig. 1A).

A variety of observations support the conclusion that E2F from infected cells binds cooperatively to the two E2F sites in the E2 control region. First, a DNase I interference assay demonstrated that both binding sites on the E2 DNA were occupied in the infection-specific E2F-Ad complex (Fig. 6), and a lower concentration of infected-cell- than uninfected-cell-specific E2F was required to protect the E2 double-site DNA in footprint assays (Fig. 2). Second, the binding was sensitive to both the relative orientation of the two E2F sites and their relative positions on the DNA helix (Fig. 9). Third, formation of the doubly occupied E2 DNA complex was not first order with respect to extract concentration (Fig. 7). The nonlinear dependence is most consistent with a second-order reaction in which two factors must participate to form the complex on E2 DNA. Fourth, formation of the doubly occupied E2F complex appears to be greatly favored over formation of the singly occupied E2F complex. Finally, off-rate analysis of unfractionated extracts showed that the doubly occupied E2F complex had a long bound lifetime, whereas no similar complex was detected in uninfected-cell extracts (Fig. 5).

Using E2F from infected cells, Yee et al. (22) reported that less E2 double-site DNA than DNA containing a single E2F site was required to prevent formation of a doubly occupied complex. This observation also fits with cooperative binding.

The mechanism underlying cooperative binding by E2F molecules is unclear. Possibly, cooperativity results from protein-protein contacts between E2F molecules on E2 DNA. Alternatively, binding by the first E2F molecule might change the structure of E2 DNA to increase the affinity of E2F for the second recognition site. It is also conceivable that both mechanisms contribute to the cooperativity.

Several lines of evidence indicate that the adenovirus-specific form of E2F is a modified version of a cellular form. E2F activities from infected and uninfected cells copurified through two high-resolution chromatographic separations (Fig. 10). When cross-linked to an E2F site, both the infection-specific and normal cellular E2F activities generated photoproducts that comigrated in an SDS-containing polyacrylamide gel (Fig. 11A and C). Preparations of E2F containing the infection-derived and normal forms of E2F produced identical footprints (Fig. 2), and they each bound to four oligonucleotides related only by their E2F sites (data not shown), indicating that both activities bind to the same site. Therefore, it seems most likely that an adenovirus

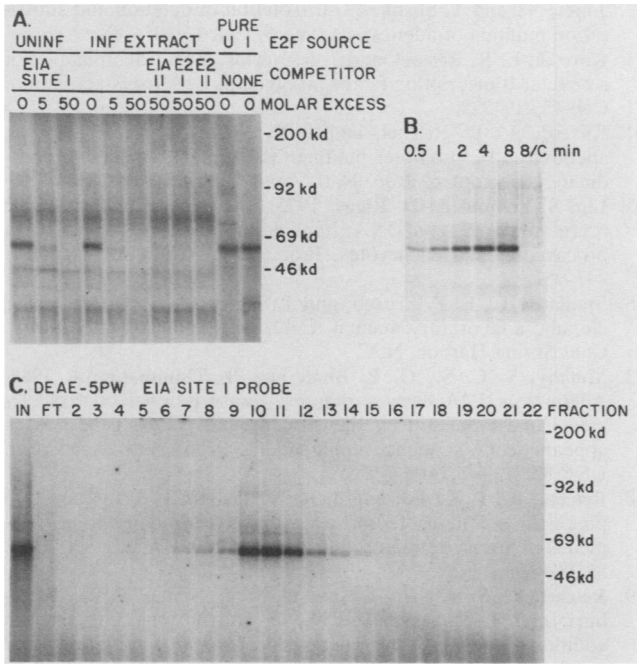

FIG. 11. UV cross-linking of E2F activities to E1A site I DNA. Binding reactions were carried out for 1 h, reaction mixtures were UV irradiated for 4 min, and photoadducts were analyzed by electrophoresis in SDS-containing polyacrylamide gels. (A) Comparison of cross-linked products produced by using E2F from uninfected or infected HeLa cells. E2F from nuclear extracts or partially purified and concentrated E2F (pure U, partially purified from uninfected cells; pure I, partially purified from infected cells) was cross-linked to ^{32}P-labeled E1A site I DNA in either the absence or presence of unlabeled competitor DNA included at the molar excess relative to probe indicated above each lane. Molecular size designations (in kilodaltons [kd]) mark the positions of unlabeled marker proteins. (B) Time dependence of cross-linking of partially purified E2F to ^{32}P-labeled E1A site I DNA. The time of UV irradiation is indicated at the top of each lane. Lane 8/C contained a 50-fold molar excess of unlabeled competitor DNA and was irradiated for 8 min. (C) Copurification of the cross-linked complex and E2F activities. Fractions from the DEAE-5PW profile displayed in Fig. 4 were cross-linked to ^{32}P-labeled E1A site I DNA, and the resulting complexes were analyzed by electrophoresis in an SDS-containing polyacrylamide gel.

product induces posttranslational modification of one of the cellular forms of E2F.

In some experiments, the total level of E2F bound to a single-site DNA increased severalfold during adenovirus infection (data not shown), but it is difficult at this point to be certain that this reflects an increase in total E2F activity, since the efficiency with which it was extracted might differ between infected- and uninfected-cell nuclei. It seems very likely that most of the induction of E2F-Ad complex observed in infected-cell extracts is due to a qualitative alteration in binding behavior (cooperativity) rather than a quantitative change in amounts of factor. This interpretation agrees with the conclusions of Reichel et al. (18), who found that the infection-specific form of E2F accumulated in cells treated with cycloheximide to prevent new protein synthesis, beginning at 1 h after infection. Reichel et al. (18) concluded that E1A was responsible for the modification. We have shown that E4 but not E1A gene products are required to generate the infection-specific form of E2F (7a). E1A products do, however, contribute to the efficiency of the E4-mediated modification process (1, 7a, 19) but probably act indirectly by stimulating E4 expression (7a).

We can readily detect E2F activity in extracts of uninfected cells, whereas previous studies (13, 14, 18, 22) concluded there was very little E2F activity present in the absence of adenovirus infection. The failure to detect the uninfected-cell-specific form of E2F in earlier work resulted from the exclusive use of a DNA probe containing the double-site E2 sequence. This probe detected E2F from infected cells at much greater efficiency than it detected that from uninfected cells in a band shift assay (Fig. 3B), giving the impression that little E2F activity was present in uninfected cells. Probe DNAs with a single E2F binding site readily detected uninfected-cell-specific E2F (Fig. 1B and 3A). E2F present in uninfected cells could also bind to both sites in the E2 control region if the assay conditions were changed (Fig. 8).

The location of E2F binding sites suggest a role in transcription. Indeed, the sites map to domains shown to be important for transcription of both the E1A (9, 10) and E2 (11, 23) genes, although in one case alteration of a single E2F site upstream of the E2 early transcriptional start site did not affect either basal or E1A-inducible E2 transcription in transfection assays (11). Yee et al. (21) reported that the pair of E2F sites normally found upstream of the E2 early start site stimulated transcription of a test gene when the gene was transfected into cells previously infected with adenovirus. Further, in vitro transcription directed by a promoter carrying the repeated sequence was induced by a factor of six when substantially purified E2F was added to an extract from uninfected cells (21).

A single E2F site may stimulate transcription in some contexts. Kovesdi et al. (14) introduced a single E2F binding site as part of an 83-bp sequence upstream of the β-globin TATA box and found that it stimulated expression of the test gene in a virus-infected cell. However, it is difficult to extrapolate from this result to the role of the infection-specific form of E2F, since cooperative binding requires a correctly arranged pair of E2F sites.

The requirement for correctly arranged sites suggests an interesting possibility. The arrangements of E2F sites in the E1A and E2 control regions are different (Fig. 1A), and E2F from infected cells binds differently to these two sets of sites (Fig. 5). This finding suggests that E2F could function differently at the two control regions during an adenovirus infection. One observation that may bear on this point is that the accumulation of the infection-specific form of E2F is maximal at the late stage of infection (7a), and it parallels a previously noticed change in E2 transcription. A second transcriptional start site (1 kilobase downstream of the E2 early start site) is activated and predominates during the late stage of infection (4). Thus among numerous possibilities, E2F could alter the relative usage of the two E2 start sites. Because of the different arrangement of sites, the effect of E2F on E1A transcription could be unchanged by its infection-specific modification.

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