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Analysis of Human Breast Cancer Nuclear Proteins Binding to the Promoter Elements of the c-*myc* Gene

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Abstract The expression of the c-*myc* gene is essential for the proliferation of both hormone-dependent and -independent human breast cancer cells. The regulation of c-*myc* gene expression in MCF-7 (hormone-dependent, estrogen-receptor (ER)-positive) and MDA MB 231 (hormone-independent, ER-negative) human breast cancer cells differs, with the c-*myc* gene of MCF-7 but not MDA MB 231 cells being regulated at the transcriptional level by estrogen. We have shown previously that the DNAase I hypersensitive (DH) sites in the c-*myc* chromatin of hormone-dependent and -independent human breast cancer cells were similar, with the exception of DH site II_2. DH site II_2, which maps near the P0 promoter, was less sensitive in hormone-dependent than in hormone-independent cells. As DH sites generally indicate the presence of sequence-specific DNA-binding proteins, we undertook a study to identify the nuclear proteins isolated from MCF-7 and MDA MB 231 cells that bound to the P0 and P2 promoter regions of the c-*myc* gene in vitro. The studies presented here provide evidence that Sp1 and/or Sp1-like proteins bind to the P0 and P2 promoter regions of the c-*myc* gene of MCF-7 and MDA MB 231 cells. Furthermore, evidence is presented for the presence of several previously unidentified sequence-specific DNA-binding proteins binding to these promoters. The DNA-binding activities of these latter proteins differed in the nuclear extracts of the MCF-7 and MDA MB 231 human breast cancer cells.

Key words: human c-myc, transcription factors, promoters, human breast cancer, nuclear proteins

The c-*myc* protooncogene is involved in the control of cellular proliferation and differentiation [for review see Dang, 1991; Marcu et al., 1992]. *Myc* has been implicated as a transcription and replication factor. The regulation of c-*myc* expression is complex and can occur at the levels of transcription initiation [Bentley and Groudine, 1986a], transcript elongation [Bentley and Groudine, 1986b; Bentley and Groudine, 1988], and messenger RNA stability [Piechaczyk et al., 1985]. Estrogen stimulates transcription of the c-*myc* gene in estrogen-dependent (MCF-7) cells, whereas constitutive c-*myc* expression is observed in hormone-independent (MDA MB 231), estrogen-receptor (ER)-negative human breast cancer cells [Dubik et al., 1987; Dubik and Shiu, 1988]. The c-*myc* gene has a critical role in the growth of human breast cancer cells [Watson et al., 1991]. An antisense c-*myc* phosphorothioate oligonucleotide that specifically inhibits expression of the *Myc* protein inhibits estrogen-stimulated cell growth of MCF-7 cells and also has a cytostatic effect on the growth of estrogen-independent MDA MB 231 cells.

Our investigation of the molecular mechanisms associated with both hormone-regulated expression and constitutive expression of the c-*myc* gene in human breast cancer cells was initiated with an analysis of the DNAase I hypersensitive (DH) sites in the 5′ flanking region of the c-*myc* chromatin in hormone-dependent and -independent human breast cancer cells [Miller et al., 1993]. DH sites often mark the chromatin fibre for the presence of sequence-specific DNA-binding proteins associated with *cis*-acting regulatory DNA elements. We found that the DH sites of the c-*myc* chromatin of hormone-dependent and -independent breast cancer cell lines were similar but not identical. In the c-*myc* chromatin 5′ flanking region, four DH sites, I, II_2, III_1, and III_2, were found. The DH site III_2, a prominent DH site in both hormone-dependent and -independent human breast cancer cells, mapped with the P2 promoter of the c-*myc* gene.

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DH site III_2 is also a site of estrogen-regulated transcription of the human c-*myc* gene, with a *cis*-acting element responding to estrogen being found in this region [Dubik and Shiu, 1992]. However, an analysis of the DNAase I hypersensitive (DH) sites failed to show a hormone-inducible DH site in hormone-responsive c-*myc* gene chromatin of human breast cancer cells. DH site III_1 was found near the P1 promoter, while DH site II_2 was located near the P0 promoter. DH site II_2 was more accessible to DNAase I in hormone-independent ER- cells than in hormone-dependent ER+ cells. The nuclease accessibility of DH site II_2 did not correlate with promoter P0 usage. In human breast cancer cells, the P2 promoter is the strongest promoter, with P2 > P1 ≫ P0 [Miller et al., 1993].

In this report the human breast cancer nuclear proteins binding to the c-*myc* P0 (DH site II_2) and P2 promoter (DH site III_2) regions were identified. In in vitro electrophoretic mobility-shift and DNAase I footprinting assays, we found the previously reported transcription factors (e.g., Sp 1) and several heretofore unidentified sequence-specific DNA-binding proteins bound to these regions. Some of these latter proteins that were associated with DH site II_2 were in greater abundance in the MDA MB 231 cells.

MATERIALS AND METHODS

Generation of End-Labelled DNA Probes

An HaeIII/AccI (−788/−608 relative to P1) c-*myc* DNA fragment was subcloned into the SmaI site of the multicloning region of pSP73. Using restriction sites in the vector, the DNA was either digested with XhoI or BamHI (Pharmacia), Bate d' Urfé, Canada. The DNA was end-labelled with the Klenow fragment of DNA polymerase (Gibco/BRL, Burlington, Canada) and [α-^{32}P]TTP (XhoI site) or [α-^{32}P]dGTP (BamHI site). Alternatively, the DNA was labelled at the BamHI site with T4 polynucleotide kinase and [γ-^{32}P]ATP. After precipitation of the end-labelled DNA, the plasmid was digested with the opposite enzyme and purified from a 4% acrylamide gel. Aliquots of the labelled c-*myc* fragment were then digested with the restriction enzymes AspI, AluI, and NsiI (Stratagene, LaJolla, CA, New England Biolabs, Mississauga, Canada) and purified from an 8% acrylamide gel.

The AvaII/Cfr101 fragment (−1/+140 relative to P1) c-*myc* DNA fragment was subcloned into the SmaI site of the multicloning site of pBluescript. Using restriction sites in the vector, the DNA was either digested with EcoRI or BamHI and end-labelled with the Klenow fragment of DNA polymerase. After precipitation of the end-labelled DNA, the plasmid was digested with the opposite enzyme and purified from a 4% acrylamide gel. For the smaller fragment, digestion with XhoI was used.

Oligonucleotides used are as follows:

S1, 5′-TCGGGTACCCCCTGCCCCTCCCATATTCTCCC-3′ (−1,065 to −1,034);
S2, 5′-TGAGCAGGCGGGGCAGGAGGGGCGGTATCT-3′ (−774 to −745) [Lang et al., 1991];
O-II, 5′-ACATAATGCATAATACATGACTCCCCC-3′ (−677 to −651);
O-II/III, 5′-ACATAATGCATAATACATGACTCCCCCCAACAAATGCAA-3′ (−677 to −639);
O-III/IV, 5′-TCCCCCCAACAAATGCAATGGGAGTTTATTCATAACG-3′ (−656 to −620);
ME1a1, 5′-AGAACGGAGGGAGGGATCGCGCTGA-3 (+107 to +131);
ME1a2, 5′-GCCTCGAGAAGGGCAGGGCTTCTC-3′ (+65 to +88) [Moberg et al., 1992]; and
MBP-1, 5′-AGGGATCGCGCTGAGTATAAAAGCCGTTT-3′ (+118 to +146) [Ray and Miller, 1991].

The high affinity Sp1 oligonucleotide was from the Hotfoot DNAase I Footprinting Kit (Stratagene).

Isolation of Nuclear Extracts

Nuclear extracts were isolated from MCF-7 and MDA MB 231 human breast cancer cells by a modification of the Dignam method [Dignam et al., 1983]. Briefly, subconfluent cells were harvested by scraping with a rubber policeman. The cells were pelleted, washed with Isoton, and then lysed with a glass dounce homogenizer in five packed cell volumes (PCV) of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$ with 0.25% (v/v) NP40, 0.5 mM DTT, 1 mM phenylmethanesulphonyl fluoride (PMSF), 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 40 μg/ml bestatin freshly added). After low speed centrifugation the nuclear pellet was washed with five PCV and then two PCV of buffer A that did not have NP40. The nuclei were extracted by resuspending to 200 A_{260} units in buffer C (20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA with 0.5 mM DTT, 1 mM PMSF, aprotinin, leupeptin, and bestatin added as before) with the addition of 2 M KCl to a final concentration of 0.42 M. The extracted nuclei were pelleted, and the supernatant was dialysed in a microdialyser (Integrated Separation Sys-

tems) for 60 min against buffer D (20 mM Hepes, pH 7.9, 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA with freshly added 0.5 mM DTT and 1 mM PMSF). The dialysed nuclear extract was aliquoted, snap frozen on dry ice/ethanol, and stored at −80°C.

Electrophoretic Mobility Shift Assay

Nuclear extracts from MCF-7 or MDA MB 231 cells were preincubated with poly dI-dC (Sigma, Mississauga, Canada; Pharmacia) in 20 mM Hepes, pH 7.9, 0.1 M KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 20% (v/v) glycerol, 0.05% (v/v) NP40, and 0.1 mM $ZnCl_2$ on ice before the addition of 0.25 to 1 ng end-labelled DNA. For some experiments, nuclear extracts were incubated with anti-Sp1 rabbit polyclonal IgG (Santa Cruz, Biotechnology Inc., Santa Cruz, CA) prior to the addition of end-labelled DNA. After 30 min incubation on ice the DNA-protein complexes were resolved on 4% acrylamide 0.5 × TBE (45 mM Tris/borate, 1 mM EDTA) gels run at 250 volts at 4°C. The gels were dried on Whatman paper and exposed to Kodak X-ray film.

DNAase I Footprinting Assay

Nuclear extracts were incubated as described for the electrophoretic mobility shift assay, except the reaction contained 30–60 μg of crude nuclear extract and 2–5 ng of labelled DNA. The reaction was incubated at room temperature instead of on ice, and samples were treated with 0.01–0.4 units of DNAase I for 2 min. The DNAase I digestion was stopped with 1% (w/v) SDS, 0.1 M KCl, 0.02 mM EDTA, 10 μg/μl proteinase K, and 5 μg/μl tRNA and incubated for 60 min at 55°C. Samples were extracted once with phenol/chloroform/isoamyl alcohol (24/24/1) and precipitated with 2.5 volumes of ethanol. Pellets were resuspended to 10,000 cpm per microliter, and equal counts were loaded onto a 6% acrylamide/8 M urea sequencing gel.

RESULTS

Identification of Sequence-Specific DNA-Binding Proteins That Bind to DNA Fragments Spanning the DH Site II_2/P0 Region of c-*myc*

The electrophoretic mobility shift assay (EMSA) and DNAase I footprinting assay were used to identify nuclear proteins that bind in vitro to the region spanning DH site II_2 (HaeIII [−788]-AccI [−608]) (see Fig. 1). The HaeIII-AccI DNA fragment was subcloned into the plasmid pSP73. The end-labelled 203 bp XhoI-BamHI DNA fragment that contained the HaeIII-AccI fragment was incubated with nuclear extracts from MCF-7 (ER+) and MDA MB 231 (ER−) cells and subjected to nondenaturing gel electrophoresis. Several DNA-protein complexes were formed with this 203 bp DNA fragment (Fig. 2, lanes 2,3,14,15). The slow migration of the major complexes suggested that

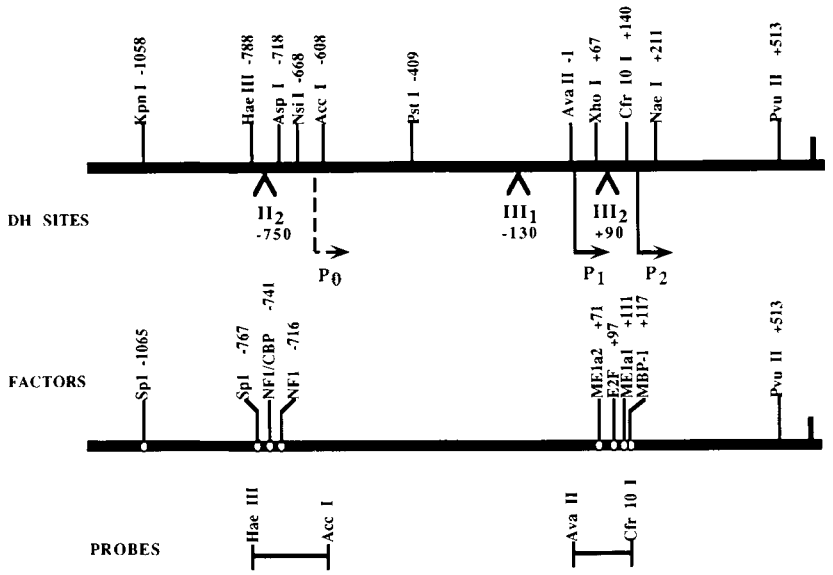

Fig. 1. Summary of previously identified in situ DNAase I hypersensitive sites and in vitro transcription factor binding sites in the human c-*myc* 5′ flanking sequences.

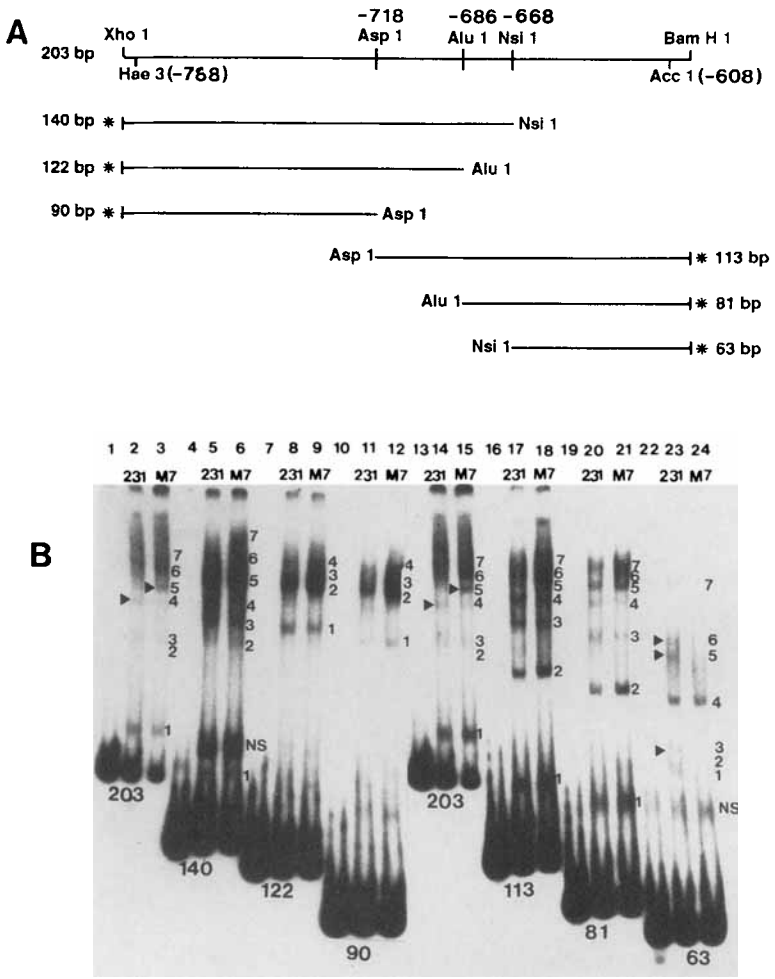

Fig. 2. Stairway assay with the 5′ flanking region of the c-*myc* gene that contains the DH II_2 site. The XhoI-BamHI DNA fragment was end-labelled separately at either end, and the fragment was shortened using internal restriction sites as described in Materials and Methods. **A:** The expected labelled fragments generated from the restriction endonuclease digestion. **B:** The DNA-protein complexes generated with the labelled fragments (1 ng) and MDA MB 231 (*lanes 2,5,8,11,14,17,20,23*) and MCF-7 (*lanes 3,6,9,12,15,18,21,24*) nuclear extracts (2–3 μg). *Lanes 1,4,7,10,13,16,19,22:* Labelled probes without added proteins. The protein-DNA complexes generated with each DNA fragment are numbered according to their mobilities relative to free DNA. NS, nonspecific protein-DNA complex.

several proteins were binding to this DNA fragment. The protein-DNA complexes were eliminated when 100-fold molar excess of unlabelled fragment was included in the incubation (data not shown). The spectra of the complexes formed with MCF-7 and MDA MB 231 nuclear extracts were similar, but differences were noted (Fig. 2, lanes 2,3,14,15 [see arrowheads]).

To locate the protein binding sites in the 203 bp DNA fragment, the Stairway assay [van Wijnen et al., 1992] was used. The XhoI-BamHI fragment was end-labelled at either end and shortened at internal restriction sites (Fig. 2). The results of this assay demonstrated that proteins were binding to sites located along the entire length of the 203 bp fragment, accounting for the complicated nature of the EMSA patterns generated with this fragment.

With the full-length fragment (203 bp) and the XhoI-NsiI (140 bp) fragment several DNA-protein complexes were detected. The more abundant slow migrating complexes were also

produced with the XhoI-AspI (90 bp) fragment (Fig. 2, lanes 11,12). These complexes could be generated by Sp1 and/or NF1/CBP (see Fig. 1) which were previously described by Lang et al. [1991] using HeLa cell nuclear extracts.

With the c-*myc* fragment labelled at the BamHI site, several DNA-protein complexes were generated with full-length (203 bp) and the AspI-BamHI fragment (113 bp) (Fig. 2, lanes 14,15,17,18). Some of the complexes formed with the 113 bp AspI-BamHI fragment appeared also to be generated with the 63 bp NsiI-BamHI region (Fig. 2, lanes 23,24; see also Fig. 6). For example, from an analysis of the complexes' electrophoretic mobilities with the various DNA fragments, complex 2 (113 bp fragment) appeared to be formed with the 81 bp (complex 2) and 62 bp (complex 4) DNA fragments. These observations suggested that the protein(s) generating complex 2 with the 113 bp DNA fragment was binding within the 63 bp NsiI-BamHI (−668 to −608) region. Nuclear proteins interacting with this region (−686 to −608) of DH site II_2/P0 have not been previously reported. MDA MB 231 and MCF-7 nuclear extracts formed several complexes with the 63 bp NsiI-BamHI region that differed in intensities (Fig. 2; compare lane 23 with 24 for complexes 3, 5, and 6; see also Fig. 6).

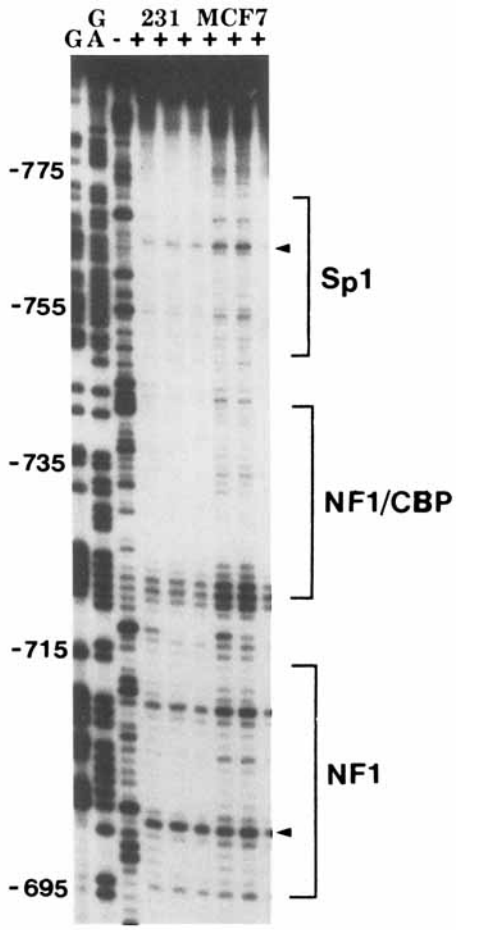

Fig. 3. DNAase I footprint analysis of the c-*myc* region −788 to −686. DNAase I footprint analysis was done as described in Materials and Methods. The sense strand XhoI-BamHI fragment was labelled at the BamHI end with Klenow. The G and G + A reactions were run in *lanes G* and *GA*, respectively. The DNAase I digested DNAs (2 ng) with (+) and without (−) nuclear extract (50 μg) from MDA MB 231 and MCF-7 human breast cancer cells are shown. The amount of DNAase I added to 40 μl reaction was as follows (left to right): 0.1, 0.2, 0.1, 0.01, 0.2, 0.1, and 0.01 units.

Association of Sp1 and NF1 With DH Site II_2

The positions of the nuclear proteins binding to the region −788 to −686 were determined by the DNAase I footprinting assay. Figure 3 shows that the DNAase I–protected regions generated by nuclear extracts from MCF-7 and MDA MB 231 cells were similar. These protected regions corresponded to the Sp1, NF1/CBP, and NF1 transcription factor binding sites previously described [Lang et al., 1991].

An oligonucleotide (S2) containing the Sp1 binding site was synthesized and used in EMSA. Figure 4A shows that MCF-7 and MDA MB 231 nuclear extracts formed a similar pattern of protein-DNA complexes with this oligonucleotide. The complexes generated with the S2 oligonucleotide were identical to those formed with an oligonucleotide containing a high affinity Sp 1 binding site. Comparable complexes (i.e., one or two predominant complexes and a faster migrating minor complex) have been observed with oligonucleotides containing variants of the Sp1 consensus sequence and different cell nuclear extracts (e.g., nuclear extracts from HeLa, NIH 3T3, C-33A, F9, and retinoblastoma cell lines Y79 and WERI-27) [Lang et al., 1991; Kim et al., 1992; Udvadia et al., 1993]. The pattern of protein-DNA complexes obtained with the Sp1 oligonucleotide and either human breast cancer cell or chicken immature erythrocyte nuclear extracts were alike (Fig. 4A, lanes 5–7). The slowest migrating predominant complexes were generated by Sp1 [Sun et al., 1992; Udvadia et al., 1993; DesJardins and Hay, 1993].

Figure 4B shows the results of competition experiments with oligonucleotides (S1, S2, and Sp1) containing Sp1 binding sequences. Each of

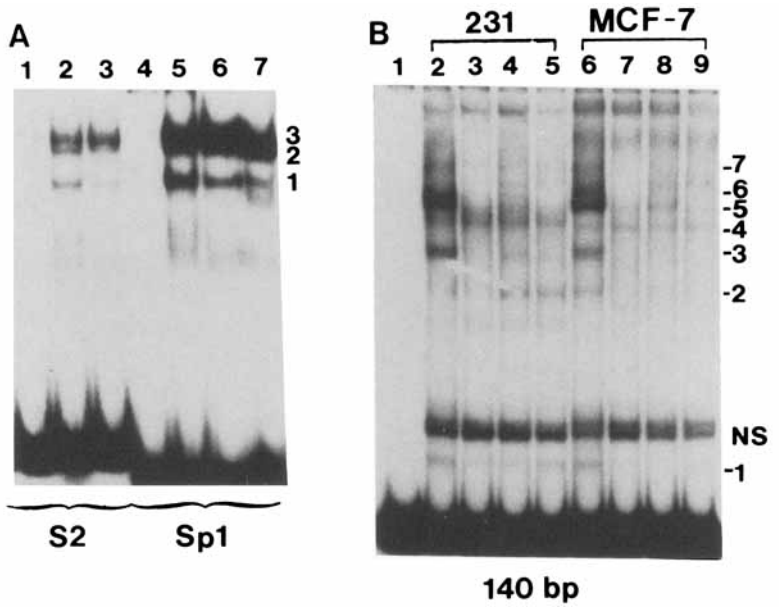

Fig. 4. Sp1 binds to the S2 DNA sequence. The oligonucleotides (1 ng) described by Lang et al. [1991] were used in EMSA with nuclear extracts (2–3 μg) from the MCF-7 and MDA MB 231 cells. **A:** Results of EMSAs with nuclear extracts from MDA MB 231 cells (*lanes 2,5*), MCF-7 cells (*lanes 3,6*), and chicken erythrocytes (*lane 7*) with S2 (*lanes 1–3*) and Sp1 (*lanes 4–7*) oligonucleotides. **B:** The XhoI-NsiI (−670) region (1 ng) of c-*myc* was end-labelled and used in EMSA with nuclear extracts (2–3 μg) from MDA MB 231 (*lanes 1–5*) and MCF-7 (*lanes 6–9*) cells. A 100-fold molar excess of S1 (an Sp1 binding sequence that is at −1,065) [Lang et al., 1991] (lanes 3,7), S2 (lanes 4,8), and Sp1 (lanes 5,9) oligonucleotides were added as specific competitors of DNA-protein complexes. Nonspecific DNA-protein complexes are shown as NS. The numbers at the right of each panel identify the protein-DNA complexes.

these oligonucleotides removed most of the complexes formed with the 140 bp XhoI-NsiI DNA fragment, providing evidence that most of the complexes forming with this 140 bp DNA fragment were due to the binding of Sp1 and/or Sp1-like proteins. The footprinting analysis suggested that the complexes remaining after the removal of Sp1 and Sp1-like proteins were generated by NF1 and CBP.

Identification of Novel Sequence-Specific DNA-Binding Proteins Associated With the DH Site II_2 Region of c-*myc*

Figure 5 shows the results of a DNAase I footprinting assay with the 113 bp AspI-BamHI DNA fragment and nuclear extracts from the MCF-7 or MDA MB 231 cells. The protected regions generated with the MCF-7 and MDA MB 231 nuclear extracts were similar. Protected region I was located between the AspI and NsiI sites and contained the AluI site. Protected regions II–IV were placed between the NsiI and AccI sites (see Fig. 10).

To tentatively assign the protein-DNA complexes formed with the 63 bp NsiI-BamHI DNA fragment to specific footprints, oligonucleotides were synthesized that encompassed footprints II–IV detected in the NsiI-AccI region (Fig. 6). Oligonucleotides O-II, O-II/III, and O-III/IV spanned protected regions II, II-III, and III-IV, respectively. These oligonucleotides were used in EMSA alongside the 63 bp NsiI-BamHI DNA fragment. The EMSA results indicated that each protected region formed protein-DNA complexes. Slow migrating high molecular weight protein-DNA complexes were generated with the oligonucleotides O-II and O-II/III (Fig. 6). Complexes 1, 2, and 3 were generated with oligonucleotide O-III/IV (Fig. 6). In other EMSAs with these oligonucleotides we observed that complexes 3–10 formed with oligonucleotide O-II/III, and complexes 1–4 and high molecular weight complexes formed with oligonucleotide O-III/IV. The protein-DNA complex that was clearly of different abundance between MDA MB 231 and MCF-7 nuclear extracts using the 63 bp DNA fragment was complex 3. Our results suggest that the protein(s) binds to the protected regions III and IV.

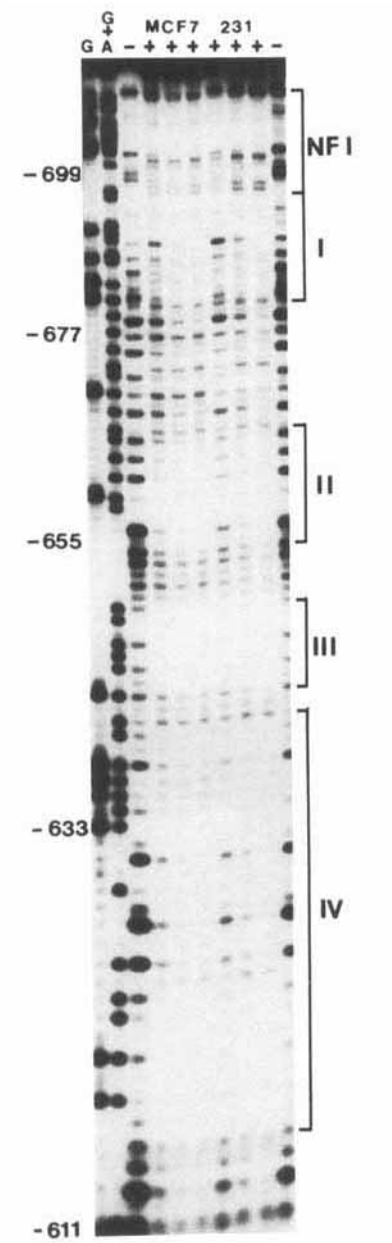

Fig. 5. DNAase I footprint analysis of the HaeIII-AccI c-*myc* region. DNAase I footprinting was done as described in Materials and Methods. Sense strand XhoI-BamHI fragment was labelled at the BamHI end with Klenow. The G (*lane G*) and G + A (*lane G + A*) reactions were run. The DNAase I digested DNA (3 ng) with (+) and without (−) nuclear extract (60 μg) from MDA MB 231 and MCF-7 cells is shown. The amount of DNAase I added to 40 μl reaction was as follows (left to right): 0.2, 0.01, 0.1, 0.2, 0.01, 0.1, 0.2, and 0.1 units. The Roman numerals indicate the protected regions.

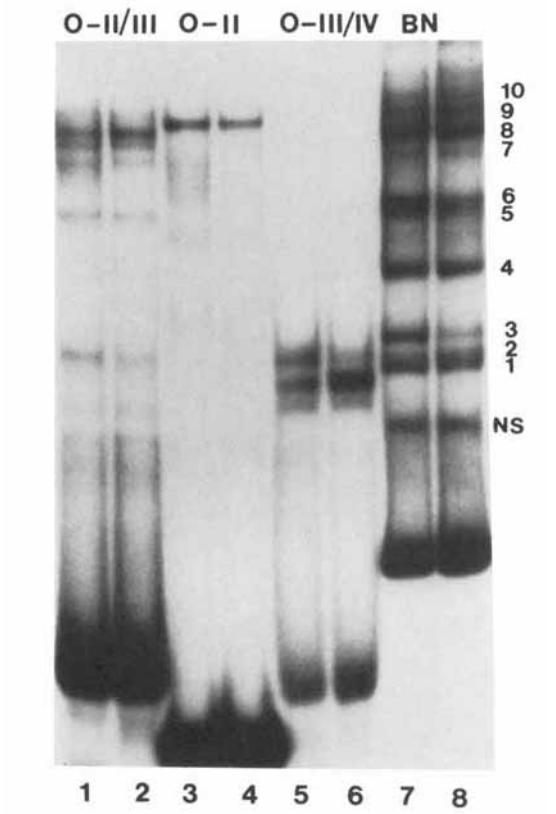

Fig. 6. Identification of the complexes formed with the various DNAase I protected regions in the c-*myc* 5′ flanking sequences −670 to −608. Nuclear extracts (10 μg) from MDA MB 231 (*lanes 1,3,5,7*) and MCF-7 (*lanes 2,4,6,8*) were incubated with the oligonucleotides (1 ng) O-II/III (footprints II and III) and O-III/IV (footprints III and IV) or DNA fragment NsiI (−670)-BamHI. Nonspecific DNA-protein complexes are denoted as NS. The numbers at the right of each panel identify the protein-DNA complexes.

Identification of Nuclear Proteins That Interact With DNA Sequences in the DH Site III_2 Region of c-*myc*

The P2 is the major c-*myc* promoter used in hormonally dependent and independent human breast cancer cells [Miller et al., 1993]. It is also a site of estrogen-regulated transcription, with the *cis*-acting element responding to estrogen being localized in the +25 to +141 region of the c-*myc* gene [Dubik and Shiu, 1992]. This 116 bp region of the c-*myc* gene also maps with the DH site III_2 (see Fig. 1). We identified proteins of human breast cancer nuclear extracts binding specifically to this region. Initially, the DNA fragment EcoRI-BamHI containing the region −1 to +140 was used for EMSA (Fig. 7). The spectra of the complexes formed with MCF-7 and MDA MB 231 nuclear extracts were similar.

The AvaII-Cfr101 DNA fragment has binding sites for several sequence-specific DNA-binding proteins, including ME1a1, ME1a2, E2F, and MBP-1 (see Fig. 1). Oligonucleotides containing the binding site of these proteins were used in EMSA with nuclear extracts from MCF-7 and

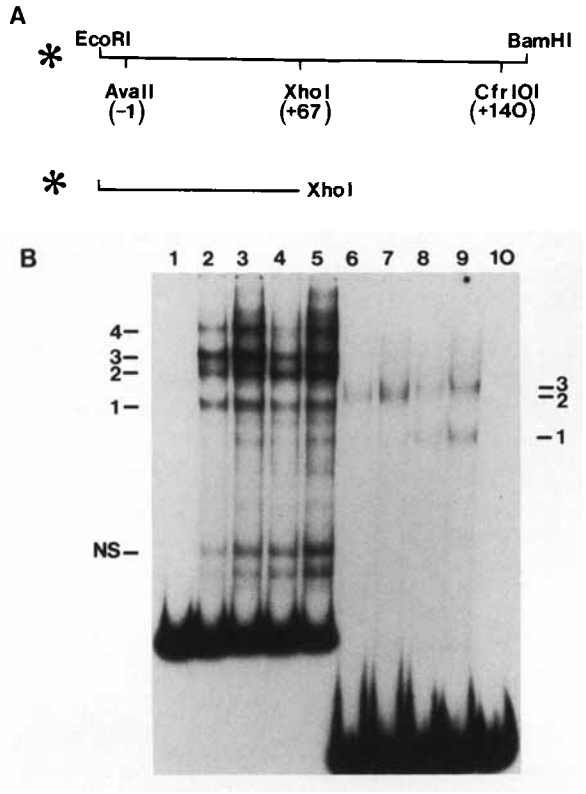

Fig. 7. Interactions between the DH site III_2/P2 promoter region and proteins from human breast cancer cell nuclear extracts. **A:** EcoRI-BamHI (labelled DNA fragment in B, *lanes 1–5*) and EcoRI-XhoI (+67) (labelled DNA fragment in B, *lanes 6–10*) DNA fragments (1 ng) were labelled as shown by the asterisks. **B:** Zero μg (*lanes 1,10*), 2 μg (*lanes 2,4,6,8*), or 4 μg (*lanes 3,5,7,9*) nuclear-extracted protein from MCF-7 (lanes 4,5,8,9) and MDA MB 231 cells (lanes 2,3,6,7) were incubated with the end-labelled DNA fragments and subjected to EMSA as described in Materials and Methods. NS, nonspecific protein-DNA complex. Numbers on the left and right of the panel identify protein-DNA complexes.

MDA MB 231 cells (Fig. 8). Specific complexes were formed with ME1a1, ME1a2, and MBP-1. We were unable to form complexes with the E2F oligonucleotide. Oligonucleotides ME1a1, ME1a2, and S2 (Sp1 binding sequence in the P0 promoter region) formed three complexes with identical electrophoretic mobility (complexes 1, 2, and 3). Since Sp1 oligonucleotides formed the same three complexes (Fig. 4), these observations suggest that Sp1 and/or Sp1-like proteins were binding to these oligonucleotides. Further, the ME1a1 (AGGGAGGGA) and ME1a2 (AGGGCAGGG) sequences are similar to that of the Sp1 consensus binding sequence [(G/T)(G/A)GG(C/T/A)(G/T)(G/A)(G/A)(G/T)] [Sun et al., 1992; Faisst and Meyer, 1992]. The human protein MAZ or ZF87 also binds to the ME1a1 element. However, this protein binds weakly, if at all, to a high affinity Sp1 sequence or ME1a2 [Bossone et al., 1992; Pyrc et al., 1992]. Since complexes 1–3 are formed with the oligonucleotides ME1a1, ME1a2, S2, and high affinity Sp1 (Figs. 4, 8), it is unlikely that these complexes contained MAZ/ZF87. To determine which of the complexes contained Sp1, nuclear extracts from MDA MB 231 cells were incubated with anti-Sp1 antibodies. Figure 8B shows that the presence of anti-Sp1 antibodies reduced the abundance of complexes 2 and 3 formed with oligonucleotide ME1a2. Identical results were obtained when nuclear extracts from MCF-7 cells were used (not shown). Similarly, anti-Sp1 antiserum reduced the abundance of slow migrating complexes formed with HeLa nuclear-extracted protein and ME1a1 oligonucleotides (called CT-I_2) [DesJardins and Hay, 1993]. These results provide evidence that Sp1 present in the nuclear extracts of MCF-7 and MBA MD 231 formed complexes 2 and 3 with oligonucleotides Sp1, S2, ME1a1, and ME1a2.

In competition experiments oligonucleotides ME1a1 or ME1a2 efficiently competed for most of the complexes that formed with the EcoRI-BamHI fragment shown in Figure 7 (data not shown). The results of this experiment were similar to those shown in Figure 4B when either S1, S2, or Sp1 oligonucleotides were used as competitors for the formation of complexes to the 140 bp DNA fragment. Note that the mobility of the complexes formed with the 140 bp XhoI-NsiI and EcoRI-BamHI DNA fragments were similar (compare Fig. 4B with Fig. 7B). These observations suggest that the principal nuclear protein binding to these DNA fragments was Sp1/Sp1-like proteins.

The previously identified sequence-specific DNA-binding proteins that bind to the AvaII-Cfr101 DNA fragment have been found between the XhoI and Cfr101 sites. To find whether proteins were recognizing DNA sequences localized between AvaII and XhoI, and end-labelled EcoRI-XhoI fragment was used in EMSA with the breast cancer cell nuclear extracts. Figure 7B shows that MCF-7 and MDA MB 231 nuclear extracts formed several protein-DNA complexes with this DNA fragment.

Identification of DNA-Binding Protein Sites in the DH Site III_2 Region of c-*myc*

Using the DNAase I footprinting assay, proteins from both breast cancer cell nuclear ex-

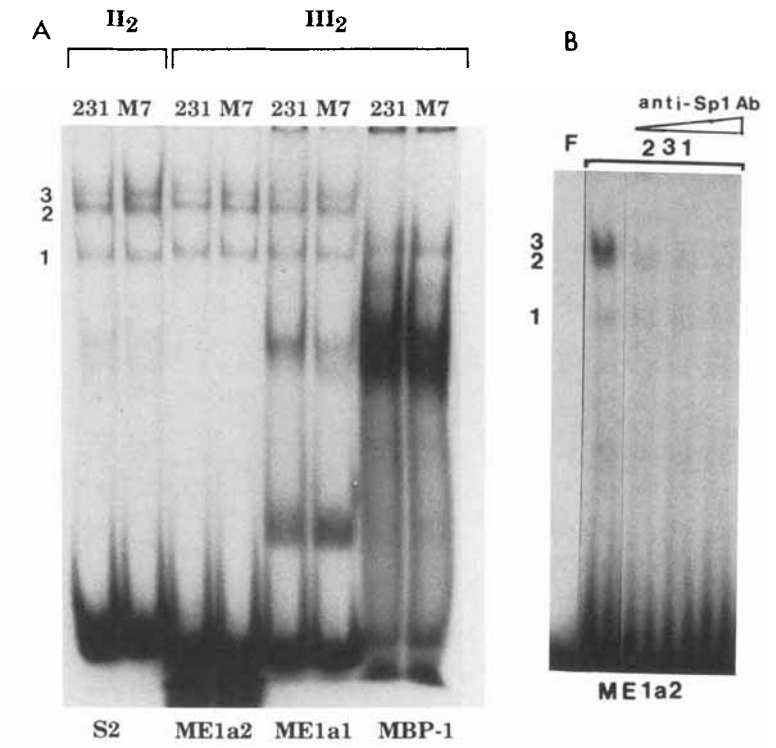

Fig. 8. EMSA with oligonucleotides S2, ME1a1, ME1a2, and MBP-1 and human breast cancer cell nuclear extracts. **A:** The oligonucleotides (1 ng) S2, ME1a1, ME1a2, and MBP-1 were used in EMSA with nuclear extracts (4 μg) from human breast cancer cell lines MDA MB 231 (231) and MCF-7 (M7). **B:** Oligonucleotide ME1a2 (1 ng) was used in EMSA with a nuclear extract from MDA MB 231 cells. The lanes from right to left contain labelled oligonucleotide alone (F), labelled oligonucleotide incubated with nuclear extract (10 μg), and labelled oligonucleotide incubated with nuclear extract that was preincubated with anti-Sp1 antibody (0.5, 1.0, and 2.0 μg). The numbers on the left of the panels identify the protein-DNA complexes.

tracts were found to interact with sequences located in the AvaII-Cfr101 c-*myc* DNA (Fig. 9). Protected region II contained the ME1a2 sequence, and protected region III had the binding sites for E2F, ME1a1, and MBP-1 (Fig. 10). Two in vitro DH sites were noted between footprints II and III. No major differences between cell lines were observed in these footprints. Footprint I spanned the region +49 to +63 that is 5′ to the XhoI site at +67 (Fig. 9). In vitro DH sites were observed 5′ to footprint I.

DISCUSSION

We have identified sites in the 5′ flanking region of the human c-*myc* promoters P0 and P2 to which human breast cancer nuclear sequence-specific DNA-binding proteins bind (summarized in Fig. 10). The EMSA data indicate that the activities of most proteins binding directly to the P0 and P2 promoters are similar in MCF-7 (ER+) and MDA MB 231 (ER−) human breast cancer cells. Nuclear-extracted proteins from the breast cancer cells protected Sp1 and NF1/CBP binding sites in the P0 promoter region. Further, the breast cancer cell nuclear extracts had proteins binding to DNA sequences (−694 to −607) 3′ to the NF1 binding sites. Proteins binding to this region of the human c-*myc* P0 promoter have not been previously reported. It was in this region of the P0 promoter (protected regions III and IV) that differences in the activities of nuclear proteins from MCF-7 and MDA MB 231 cells were observed, with the level of a DNA-binding protein(s) interacting with the in vitro DNAase I–protected regions being greater in MDA MB 231 nuclear extracts. The variations in the abundance of these factors may contribute to the differential sensitivity of DH site II_2 in these breast cancer cells. Another possibility is that the differential nuclease sensitivity of DH site II_2 in MCF-7 and MDA MB 231 cells reflects differences in protein-protein interactions between the P0 promoter bound proteins and P2 promoter bound proteins [Cvekl and Paces, 1992].

Except for footprints I and IV, we were unable to match the sequences of these footprints in the P0 promoter with binding sequences of known

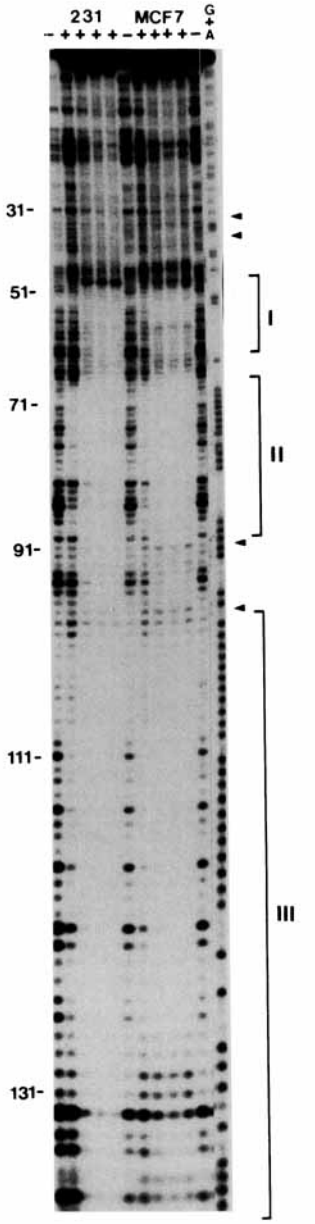

Fig. 9. DNAase I footprint analysis of the AvaII (−1)-Cfr101 (+140) c-*myc* region. DNAase I footprinting was done as described in Materials and Methods. The EcoRI-BamHI DNA fragment was labelled at the BamHI (see Fig. 7). The G + A reaction was run in the last lane. The DNAase I digested DNA with (+) and without (−) nuclear extract (20 μg per ng DNA) from MDA MB 231 and MCF-7 cells is shown. The amount of DNAase I added to 50 μl reaction was as follows (left to right): 0.2, 0.4, 0.2, 0.1, 0.01, 0.1, 0.4, 0.2, 0.1, 0.01, and 0.1 units. The arrowheads show the DNAase I hypersensitive sites of the complex. The Roman numerals indicate the protected regions.

factors [Faisst and Meyer, 1992]. Footprint I has the sequence AGATAG that binds the GATA family of transcription factors. Footprint IV contains the sequence TTATTCAT that corresponds to the GHF-1/GHF-5/GHF-7 consensus site. Pit-1, which is involved in activation of the growth hormone gene, also binds to a similar sequence [Schaufele et al., 1990]. It remains to be determined whether the breast cancer nuclear proteins binding to footprints I or IV are members of the GATA or GHF family of proteins.

The P2 promoter is the preferred promoter of human breast cancer cells [Miller et al., 1993]. The EMSA results indicate that the nuclear extracts of MCF-7 and MDA MB 231 human breast cancer cells had a similar level of nuclear proteins binding to the P2 promoter region (+49 to +140). The majority of these P2 promoter protein binding sites have been previously reported [Marcu et al., 1992], with the exception of proteins binding to DNA sequences localized between +49 and +63 (footprint I, Fig. 9). Both MCF-7 and MDA MB 231 nuclear-extracted proteins protected the ME1a1, ME1a2, E2F, and MBP sites (footprints II and III, see Fig. 10). Although we observed that the E2F site was protected, we were unable to obtain shifts in EMSA with the E2F oligonucleotide. The protein-DNA complexes produced with the ME1a1 and ME1a2 oligonucleotides and human breast cancer nuclear extracts were identical to those generated with Sp1 binding DNA sequences, S2 and Sp1. Further, nuclear extracts from chicken erythrocytes and the human breast cancer cells generated similar shifts in EMSA with the Sp1 oligonucleotide, and an anti-Sp1 antibody prevented the formation of the slowest migrating complexes with the ME1a2 oligonucleotide (complexes 2 and 3 in Fig. 8B). These results together with those of DesJardins and Hay [1993] provide support for Sp1 binding to ME1a1 and ME1a2 sequences. An analysis of the S2, ME1a1, and ME1a2 DNA sequences predicts that these sequences will have weak to medium affinity for Sp1 [Letovsky and Dynan, 1989; Lang et al., 1991; Sun et al., 1992]. None of these Sp1 binding sites are high affinity.

Dubik and Shiu [1992] localized the *cis*-acting DNA element in the 5′ flanking region of c-*myc* responding to estrogen to a 116 bp region (+25 to +141). The P2 promoter was required for estrogen-regulated transcription. The 116 bp region does not contain a classical estrogen response element (ERE), but an ERE half site (GGGCA) is present in the ME1a2 region (see Fig. 10). If the ER was to bind to this half ERE, then it would be in competition with Sp1. However, ER is unable to bind to the DNA sequences in this region (Dubik and Shiu, personal commu-

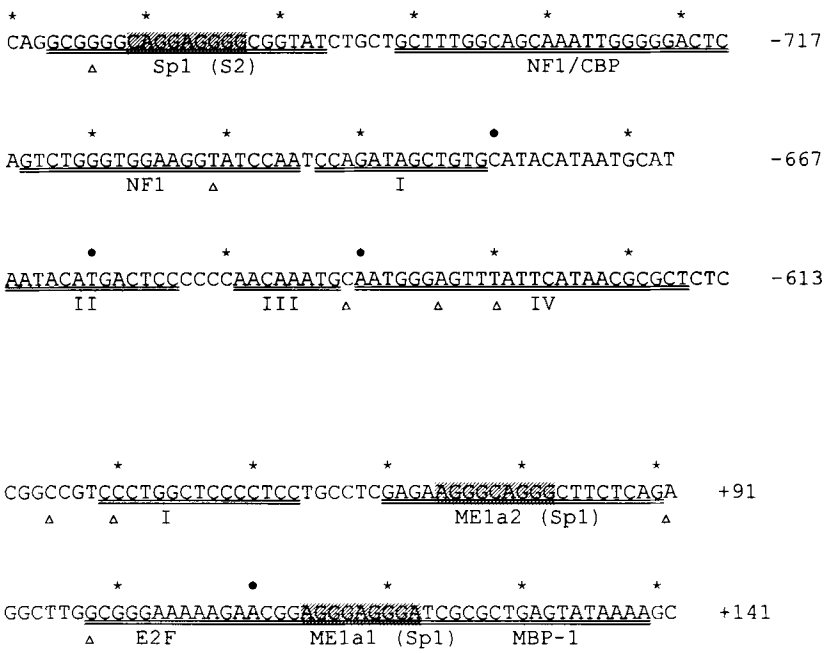

Fig. 10. Summary of data from studies of nuclear proteins binding to the c-*myc* DH site II_2 and III_2 regions and mapping of DNAase I hypersensitive sites in c-*myc* gene chromatin of human breast cancer cells. Double underlines indicate the DNAase I–protected regions, and the arrowheads show the positions of the DNAase I hypersensitive sites in the complexes. The Sp1 binding motif is shadowed. Roman numerals correspond to those shown in Figs. 5 and 9.

nication), and our studies on the chromatin structure of the c-*myc* gene of hormone-dependent and -independent human breast cancer cells failed to detect a hormone-induced DH site which would have been an indication that ER binds to the 5′ flanking DNA sequences of the c-*myc* gene [Gilbert et al., 1992]. We speculate that in hormone-dependent breast cancer cells the ER is associated with another DNA-binding protein, perhaps Sp1 and/or NF1 [Schule et al., 1988; Gorski et al., 1993], and functions as a coactivator [Sukovich et al., 1994]. This model is analogous to the proposed mechanism by which the retinoblastoma gene product positively regulates the transcriptional activity of Sp1 that is bound to the retinoblastoma control element motif [Kim et al., 1992; Udvadia et al., 1993]. Recently, Krishnan et al. [1994] have shown that in MCF-7 cells a complex formed between the estrogen receptor and Sp1 mediates the estrogen-induced expression of the cathepsin D gene. It is interesting to note that the cathepsin D gene does not have a classical ERE but is organized similarly to the c-*myc* gene with a half ERE positioned next to an Sp1 binding site, with 23 nucleotides separating the two motifs. Further, the cathepsin D half ERE motif (in bold) is nested in a sequence TGGGCGG**GGGCA** that is also a binding site for Sp1 (underlined).

DesJardins and Hay [1993] demonstrated that Sp1 (or Sp1-like protein) binds to a cluster of CT elements at −151 to −102. DH site III_1 is located in this region next to the P1 promoter. The retinoblastoma control element motif, which is an Sp1 binding site, is at −84 to −77 [Kim et al., 1992; Udvadia et al., 1993]. Together these results imply that Sp1 has a pivotal role in the activity of the three c-*myc* promoters in hormone-dependent and hormone-independent breast cancer cells. Sp1 binding at these three sites could also have a role in bringing factors associated with these regions into spatial proximity. Sp1 bound at DNA sites separated by 1.8 kb will interact, resulting in the looping out of the intervening DNA sequences [Mastrangelo et al., 1991; Pascal and Tjian, 1991; Su et al., 1991]. Thus, Sp1 associated with DH sites II_2, III_1, and III_2 may interact, resulting in a clustering of many transcription factors.

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