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ARTICLES

Analysis of the Chicken DNA Fragments That Contain Structural Sites of Attachment to the Nuclear Matrix: DNA–Matrix Interactions and Replication

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Abstract Ten short DNA fragments have been selected from a library of the nuclear matrix–attached DNA (nmDNA) from chicken erythrocytes by their ability to hybridize with the fraction of chicken replication origins isolated by nascent DNA strand extrusion. The primary structure of these fragments has been determined. Five of the sequences contained a topoisomerase II recognition site. Most of the studied DNA fragments also have a common eight-nucleotide motif, GCAGACCG/A. A sequence-specific DNA-binding protein with a MW of 55 kDa that interacted with this motif has been identified. Some of the cloned DNA fragments promoted an increased level of transient plasmid replication in transfected chicken cells. The ability of plasmid bearing nmDNA fragments to replicate correlated directly with their ability to target plasmids to the nuclear matrix compartment. J. Cell. Biochem. 79:1–14, 2000.

Key words: nuclear matrix; DNA–protein interactions; DNA replication

The data concerning different types of association of DNA with the nuclear matrix have been accumulated during the past few years [Smith et al., 1984; van der Velden et al., 1984a, 1984b; Razin, 1987; Cook, 1989] [reviewed in Razin, 1996]. In functionally active nuclei, most of the specific interactions of DNA with the nuclear matrix elements are connected with transcription [Razin, 1987; Cook, 1989]. We have previously demonstrated that these interactions disappear during differentiation of chicken erythroid cells, leading to termination of transcription in the nuclei of mature erythrocytes [Razin et al., 1985; Farache et al., 1990]. However, a specific set of DNA sequences remain associated with the nuclear matrix in inactive nuclei of mature chicken erythrocytes [Razin et al., 1985; Farache et al., 1990] and spermatocytes [Kalandadze et al., 1990]. Interestingly, the positions of these attachment sites in the domain of chicken alpha-globin genes are similar in erythrocytes, fibroblasts, and spermatocytes [Kalandadze et al., 1990]. We designated them as permanent or structural attachment sites. Further studies demonstrated that the permanent attachment sites, which have also been mapped in active cells using a topoisomerase II–mediated DNA loop excision protocol [Razin et al., 1991a], were located near replication origins on the DNA chain [Razin et al., 1986].

The organization of replication origins in higher eukaryotes is highly controversial. Although several replication origins have been mapped with a precision of one to several kilobases [Razin et al., 1986; Handeli et al., 1989; Vassilev et al., 1990; Berberich and Leffak, 1993], other data indicate that replication may initiate within a broad zone rather than at specific sites in the dehydrofolate reductase gene [Vaughn et al., 1990], and the chicken lysozyme gene [Phi-van et al., 1998].

Moreover, in *Xenopus* embryos, replication initiates at random sequences but at regular intervals in the ribosomal DNA [Hyrien and

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Méchali, 1993], and the same pattern was observed in the interaction of the rDNA cluster with the *Xenopus* nuclear matrix [Hair et al., 1998], suggesting that three-dimensional organization of the genome, and hence sequences involved in DNA attachment to the nuclear matrix, play a role in the regulation of replication. Other studies also show that nuclear architecture determines the sites of replication initiation in *Xenopus* egg extracts [Lawlis et al., 1996].

Several published reports concern the DNA sequences that promote autonomous replication of plasmids in cells of higher eukaryotes. Both fairly large (>12 kbp) [Heinzel et al., 1991; Masukata et al., 1993] and small (<1 kb) DNA fragments [Landry and Zannis-Hadjopoulos, 1991; Wu et al., 1993] can transiently support autonomous replication in mammalian cells.

In the present study, we have cloned several hundred short DNA fragments permanently attached to the nuclear skeleton. Approximately half of these fragments were preferentially represented in the sample of short double-stranded nascent DNA fragments extruded from short replication loops. Several fragments with a size of 100–500 bp were sequenced and analyzed. We have identified six DNA fragments that interact with sequence-specific DNA-binding proteins, and found that some of them enhance transient autonomous replication of plasmids in cultured chicken erythroblasts.

MATERIALS AND METHODS

Isolation of nuclear matrices and DNA attached to the nuclear matrix was carried out using high salt extraction of nuclease-treated nuclei as described [Razin et al., 1986]. Nuclear matrices from transiently transfected HD3 cells were isolated as described [Cockerill and Garrard, 1986]. In short, nuclear matrices were prepared by incubation of 1 OD_{260} of isolated nuclei with 50 U each of EcoRI, EcoRV, SpeI, and XhoI restriction endonucleases at 37°C for 3 h and subsequent extraction with 2 M NaCl. Nuclear matrices were then digested by overnight treatment with sodium dodecyl sulfate (SDS) and proteinase K. Non–matrix-associated (cleaved-off) DNA was compared to the matrix-bound DNA fraction by electrophoresis in agarose gels followed by hybridization with the pTZ19 probe and autoradiography. The data were quantified by scanning on a Shimazu scanning densitometer. The data represent an average of three independent experiments.

The MAR rebinding assay was carried out as described [Cockerill and Garrard, 1986]. In short, nuclear matrices were prepared by incubation of isolated nuclei from HD3 cells with DNase I and subsequent extraction with 2 M NaCl. Fifty nanograms of end-labeled DNA fragments and unlabeled competitor DNA (sonicated salmon sperm DNA) were added to the nuclear matrix preparations isolated from 5×10^7 cells, and incubated at room temperature for 2 h; then the nonbound DNA was removed by repeated washing with the incubation buffer. Nuclear matrices were then digested by overnight treatment with SDS and proteinase K. Matrix-bound DNA was purified by phenol extraction, and the initial complete set of labeled DNA fragments was compared to the matrix-bound DNA fraction by electrophoresis polyacrylamide gels followed by autoradiography. In some experiments, the oligonucleotide binding site for F326 was used as a competitor.

Cloning of DNA Fragments Permanently Attached to the Nuclear Matrix

Before cloning, DNA samples were size fractionated by preparative polyacrylamide gel electrophoresis. The eluted 100–500-bp-long DNA fragments were treated with Pfu polymerase I (Stratagene, La Jolla, CA) to produce blunt ends necessary for ligation of synthetic octanucleotide BamHI linker. Before ligation, both linker and DNA samples were kinased. The ligation was carried out as described [Maniatis et al., 1982]. After separation from the nonligated linker, the samples were digested with BamHI, separated from the cleaved-off linker by gel filtration, and ligated with the BamHI-pTZ19 vector.

Replication origin fraction was isolated by nascent DNA strand extrusion as described elsewhere [Zannis-Hadjopoulos et al., 1981].

DNA electrophoresis in polyacrylamide and agarose gels, DNA transfer onto nylon filters, [^{32}P]-labeling of DNA, and hybridization experiments were carried out as described elsewhere [Maniatis et al., 1982].

The primary structure of DNA was determined using the Maxam-Gilbert method modified by Chuvpilo and Kravchenko [1983].

Gel retardation experiments were carried out as described in Fried and Crothers [1981].

Chicken hepatocytes and embryonic fibroblasts served as a source of nuclear proteins from resting and proliferating cells, respectively. Nuclear extracts were prepared using the method of Plumb et al. [1986]. Nuclear matrix extracts were prepared according to Hofmann and Gasser [1991]. The protein binding experiments were carried out in a buffer containing 20 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 5% glycerol, 0.1 mg/ml poly(dAdT), 0.1 mg/ml protein extract, and synthetic oligonucleotide binding site for the F326 protein (5′-GATCCTCACTGCAGACCGCTTCG-3′) where indicated.

DNase I footprinting experiments were carried out as described in Plumb et al. [1986].

South-Western blotting and hybridization analysis was performed as described in Miskimins et al. [1985]. The blot was hybridized with the synthetic oligonucleotide probe containing the recognition site for F326 (5′-GATCCTCACTGCAGACCGCTTCG-3′).

Transient replication assay has been carried out as described in Landry and Zannis-Hadjopoulos [1991]. Briefly, logarithmically growing cultured chicken erythroblast cells (line HD3) [Beug et al., 1979] were transfected with the mixture of 3 mg of pTZ19 and pTZ19 with inserted nmDNA fragments by the DEAE-dextran (Diethylaminoethyl-dextran) method. After 24 or 48 h, cells were harvested, and low molecular weight DNA was isolated using the method of Hirt [1967]. A part of DNA was directly transfected into *Escherichia coli* or separated by SDS electrophoresis, blotted, and hybridized with the nmDNA fragments; the other part was subjected to analysis after treatment with the DpnI restriction endonuclease.

Semiconservative replication assay was carried out as described in Landry and Zannis-Hadjopoulos [1991]. The HD3 cells were transfected as described above and then were incubated in the growth medium containing 12.5 mg/ml bromodeoxyuridine for 24 and 48 h. Hirt supernatants were applied to a neutral CsCl gradient (initial refraction index 1.4150) and centrifuged at 65,000 rpm for 16 h. Fractions were collected starting from the bottom of the tubes, and the refractive index of every third fraction was measured. Aliquots of 50 ml were taken for dot blots and electrophoresis. They were probed with the corresponding nmDNA fragments and autoradiographed.

RESULTS

Cloning and Sequencing of Short Erythrocyte nmDNA Fragments

We have previously found that DNA fragments permanently attached to the nuclear matrix (nmDNA) represent only a small fraction of unique sequences of total chicken DNA [Razin et al., 1986]. One might expect that most of these fragments have some common features that account for their anchorage to the nuclear matrix. Second, using renaturation experiments, we have demonstrated that replication origins were located near permanent sites of DNA attachment to the nuclear matrix [Razin et al., 1986]. This observation has also been supported by the data of other laboratories [van der Velden et al., 1984b; Opstelten et al., 1989]. It has also been shown in yeast cells that the nuclear matrix/scaffold attachment regions (MARs) are located in the vicinity of autonomously replicating sequences [Amati and Gasser, 1988]. This suggests that the study of DNA sequences involved in organization of the nuclear matrix attachment sites may also shed light on the structure of replication origins.

To determine the nucleotide sequences directly involved in formation of the permanent attachment sites (and possibly of replication origins), we have cloned chicken erythrocyte nmDNA (see Materials and Methods) with a size range of 100–500 bp. To study the interrelationship between this fraction and replication, we have dot-blotted individual clones onto a nitrocellulose filter and then hybridized them to a purified fraction of chicken replication origins (oriDNA) isolated from nonsynchronized cultured chicken erythroblast cells by nascent DNA strand extrusion (Fig. 1A). To control the results of hybridization, we have spotted on the same filter the filter subfragments of the domain of chicken alpha-globin genes that contain a well-characterized replication origin [Razin et al., 1986], a unique sequence, and a CR1 repeat. We have also hybridized the same filter to total chicken DNA to reveal the repetitive DNA sequences among the nuclear matrix–attached DNA fragments (Fig. 1B). All the clones containing highly repetitive elements were excluded from further analysis.

This experiment led to the conclusion that ~50% of all the unique and moderately repetitive erythrocyte nmDNA fragments were located in the vicinity of replication origins. In-

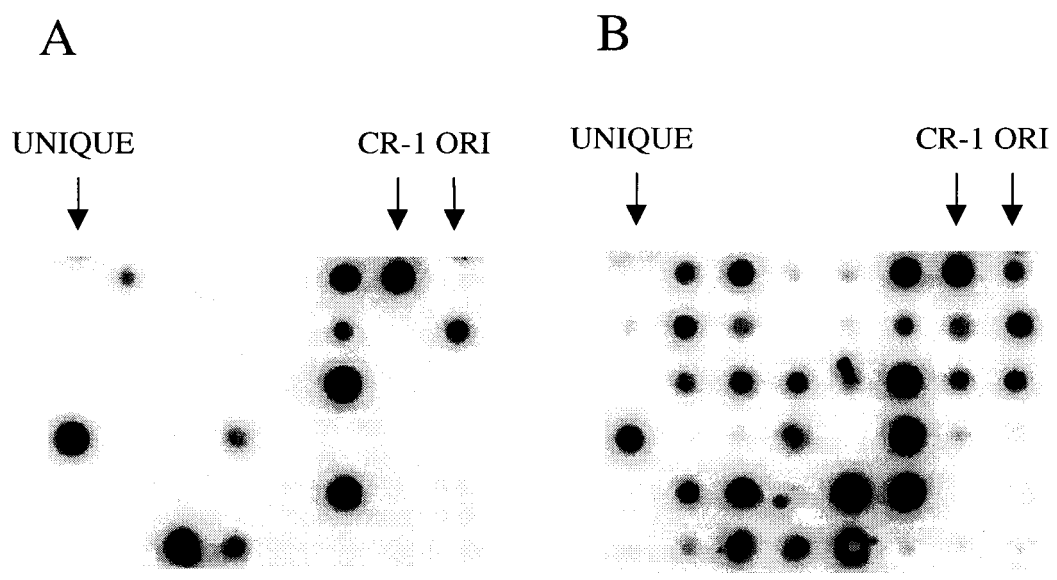

Fig. 1. Hybridization of the cloned nuclear matrix–attached DNA (nmDNA) fragments with the nick-translated total DNA (**A**) and oriDNA (**B**) probes. Individual nmDNA clones were dot-blotted onto a nitrocellulose filter. Arrows indicate the control DNA, containing: UNIQUE, a unique DNA fragment, located far from the replication origin and the nuclear matrix attachment site; ORI, a fragment, containing a replication origin; CR-1, a fragment, containing a CR-like repetitive sequence.

deed, a prominent hybridization signal was observed when these clones were probed with oriDNA but not with the total DNA probe of the same specific activity (Fig. 1). We have sequenced ten short nmDNA fragments overrepresented in the oriDNA fraction as compared to total DNA. Some of these sequences were unique, and some contained moderately repetitive DNA. These sequences are listed in Figure 2.

We did not find any pronounced similarity or consensus common to all the sequenced nmDNA fragments. However, none of them contained a substantial open reading frame; 6 of 10 sequenced fragments were relatively rich in Guanidin (G) and Cytidin (C) residues, i.e., GC-rich (although they contained short DNA stretches with high content of adenosin (A) and Thymidine (T) residues (AT content of $>80\%$)); most of the fragment had multiple imperfect internal repeats. A relatively short motif GCAGACCG/A was found in 8 of 10 sequenced DNA fragments. A computer analysis also revealed the presence of several protein binding motifs, e.g., the CO33 fragment has two putative NFIII binding sites [Pruijn et al., 1987] in opposite orientations (positions 122–144). A sequence element similar to the NFI binding site can be found in the CO77 fragment (positions 126–117 on the lower strand).

A striking similarity between the cloned nmDNA fragments was observed when we compared their sequences with the in vivo topoisomerase II consensus [Kas and Laemmli, 1992]. An 85% homology to this 18-nucleotide consensus was found in 5 of the 10 fragments. Two of the fragments contained several copies of this consensus. This observation is in agreement with the data indicating that topoisomerase II is a major component of the nuclear scaffold in metaphase chromosomes [Earnshaw et al., 1985] and that it interacts with DNA fragments that preferentially bind to the nuclear matrix in vitro [Vassetzky et al., 1989].

Direct comparison with the nuclear matrix attachment sites characterized by other authors [Goldberg et al., 1983; Opstelten et al., 1989] did not reveal strict homologies, although several of the motifs found in very short

Fig. 2. Nucleotide sequences of chicken short nuclear matrix–attached DNA (nmDNA) fragments that contain replication origins.

CO30 160 bp
ATCCGGGGGG GGTCGTGGGG GGGGGGAGTC CCCAATTCCA AGGGGGGGGT CGGAGGGTGG GGGGGGTCCC CATTCCAA
GGGGGGCCGG AAGGGGGAGG GTCCCCATTT CTCAGGGGGG GGTCGGAGGG GGGGGTCCCC ATTCCCAACG GGGGAGGG

CO326 490 bp
GGAGGTGCGG GGAAGGGGCC CGAGGAGTTG GGGTGGGGGG GGGGGGAGTT GGGGTTCTTT GGGGGTGGGT TTGGGGGT
CGGTCCGCGC TCACCCGTCA GAAGGCCGAG GAGGAGCAGC GCGGGGCCGC AGAAGGCCAT GGCGGGCCGG GCCCGGTT
GGCCTCTTCG GGGGGACCCT TCAGAGCGCT TCGGGGTTCT TCGGGTCCGG TTCGGGTCC GTTCGGCTGAC GTTCGGGT
CCGCAGACCG CTTCGGGTGT CCTTCGGGTC CGTTCGGCGG CGTTCGGGTC ACTGCAGATC ACTTCGGGTG TCCTCGGG
CGGTTCGGGT CCGTTGGCCG GCGTTCGGGT GACCGCAGAG AGCTTCGGGT CTCTTCGGGT CCGTTCGGCG GCGTTCGG
CACTGCAGAC CGCTTCGAGT
CTTTTCGGGT CCGGTTCGGG TCCGTTCGGC GGCGTTCGGG TCACTGCAGA CAACTTCGGA ACTCTTCGGG

CO33 172 bp
GATCCCTTCT TTTACAAAAT TAAGTCTGCC TAGCTCCATA TCTTTCTTTT GATTAGGTGT TATTTTAAAG GAGCTTTT
TGCCACCTAT CCTTCCAATG TTACTCTTTC TGGTTTTTTC CCCTCTTTGC ACTACTTGCA AAGATGACTT GAAGGAGT
TATTTTAGAG TG

CO34 173 bp
GAGCTCGGTT ACCCGGGGAT CCGGAATCAT AGTTTATCAG CAGCTTCCTC AAAGCAGCAC AGGAACTGCT GGGTAATC
CAGCCTGTTA CTCTAAAGAC ACCCAATGAT TTTTTCTTAC ACTGTGGACT GCTTACAAAG ACTTTCACAC TACTTCAC
GGATCCTCTA GAG

CO41 140 bp
ACTCACACAT GACAGCTCAG AAATAAAATA AAAACCTGCT TACCCCTGAA AGGCATGTAA GGACCTTAGA CAGGCAGG
TCTCTAAACA AGATACTCTT GCTTCCACTG CCAACCCTTT AATGTTATCT GGGAAGGGGT

CO56 318 bp
GCAGTCCGGT GGCACTGCAG CTGCAGCAGT GGGTGGAGAG GTAATTATCT CACTGTACTG GGTGCTCATT GAATCCCG
TAGAATATTG CATCCGGTTT GGGGCTCTCT GTTGCAACAA AAATGTCATA AATTGGAGCG AGCTCGGGGG TATGCCCC
GATGTTTCCC TGAGAGGAAA GGCTGAGGGA ACTGGGTTTG TTCAGGGTGG GGAGTAGATG GCTTTGGGGA ACCCAAGA
AACTTGCCTG TACCTGTGAG GGGGTCAGCA AGGAGTGATG CTCACTGCGC ATCCATTGCC TGTGGGAGAA GGAGGCCT

CO62 220 bp
GGAACGGAAC GGAACGGAAC GGAACGGAGC GGAGCGGAGC AGAGATGCCA CCGCCGCCGT CGGCCCGCCG TGACGCCG
CGCCGGCGGG GGCGGCGGGG AGAGCCGGGG GGGGAACCGC AGTGCCGCAG CTCCGCCGGT ACCGGAACCC CCGGAGAG
TGCGTGGCAC GGGGGGGTGC GGGAGGCGAG GGGGTGGCGG GAGGGCACGG CAGCAGGGG

CO65 318 bp
CGAGCCGGTA CCCGGGGACC CAAACTGGCA AAGGGTGCAG GTGAGGATTG TCCCAACCAG CAGTTCTGCG AGCTGTCA
CGCTGGTAGG CAGGGAGAGT TAAAAAGGAA TTAAGTGATT AGAAATGCAT AAATATTAAT TGCTTATGGA CTCCCTTT
GACACTCTCA GGTGGAGGAA GTTAATAAAT CACTGCTGGC ATTGACCCAC AAAACAATAC AGAGTGTGTC TGAGACAC
CGAGGGGAGG AGGGGGGAGG CCTTTCTGGA GCACGTGAAA GCGCTCTTTT TAGGTCAGGG AGTGACACAA CAGAGCTG

CO73 517 bp
CCCCCCAGTG GGGAGGTCCT GTGGGGGGGT TTGGGGGGGT CCTAGGGAGG TTTTGGGGGG GATAGTGGGG TTTGGGGG
TCCTATGGGG GATGGGGGGG TTCTGGAGGG GTCCAAGGGG GGATAGTGGG GTTTGGGGGG GTCCTATGGG GAATGGGG
GTCCCTGAGG GGTCCAAGGG GGGATAGTGG GGTTTGGGGG GGCTGGGTCC GTTGGGGATG GGTAGGAATG CGGCCCAC
TGAGGTCACG GGGTGACTGT GACTCTTCGG TGCCATCACG AAGCACACAG ATAGGTGGAG GTTTGCTGCC CCGTGGCT
TGCGTCAGTA TTTTGGGCAT TTGCCTGCCA GAGTGCACCC GAGGACTTTC AGTGGGCCAT TTTCCCAAGC AGTGACAA
AAAGGATTCT TGAAGCAAGC AGTAGAGCAG CTTTGGTGTT GCCGTTGATC AGTGCAGTAG CTGTGACCGT GCCGGTAT
GATAGTAGTG TCAGCAAAGT AAATGCTGTG CTGGGTA

CO77 297 bp
AAAAAAGAGC CTTCGGTGAT GGCAGGAGGG GAGACGTACG GATGGAGTCT TGCAAGAGGG ATGCTGCCAT
CCCATCCCAT AGAGGCAGAG CAAAGTGGCC ATCTTTCCTC ACCTTGTTAT TTGAATGTTG ACTAGGTGTT
CACTGTTTCA AATTTACTAT CAGGGTAATG ATAATCGTAT TCATGATTAA GGATCTTTTT TTGGCATTTG

(~10–30 bp long) MARs cloned and sequenced by Opstelten et al. [1989] could be found in our nmDNA fragments (e.g., the motif TTTTGTT can be found in one or both strands of CO33, CO41, CO56, CO73, and CO77 fragments).

nmDNA Fragments Specifically Interact With Protein Extracts From Proliferating Cells

The next series of experiments was designed to find whether specific DNA-binding proteins from nuclei of actively proliferating cells interacted with the nmDNA fragments. Protein extracts from embryonic chicken fibroblasts were incubated with the cloned fragments of nmDNA in the presence of competitor DNA. Specific binding was monitored using a band-shift assay [Fried and Crothers, 1981]. Six of the 10 nmDNA fragments, namely, CO326, CO33, CO34, CO56, CO62, and CO77, specifically interacted with protein extracts (Fig. 3A, lane 2). Some of the cloned fragments (CO33, CO34, CO56, CO77) produced several bands with mobility different from that of free DNA, indicating that they may contain several binding sites for some nuclear protein(s). All the DNA fragments that specifically interacted with the protein extracts contained the motif GCAGACCG/A. Hence, it was interesting to check whether this motif shared by the nmDNA fragments was engaged in DNA–protein interaction. We tested this idea by footprinting DNA–protein interactions within the CO326 fragment. This fragment contained this motif repeated five times within longer direct repeats that are spaced at a distance of ~60 bp.

In vitro DNase footprinting experiments with the extract from embryonic chicken fibroblasts demonstrated that all of the five sequences containing the motif interacted with a sequence-specific protein (Fig. 4). The consensus recognition sequence for this protein is T(G/C)AC(C/T)GCA(G/A)ACCGCTTC.

Competition experiments with this oligonucleotide binding site completely inhibited the interaction between the CO326 and the protein extracts (Fig. 3A, lanes 3 and 4). The binding of the protein factor that we have designated as F326 only occurred with double-stranded DNA; no single-strand-binding activity was detected (data not shown).

Some of the retarded bands were also eliminated in the CO34 and CO77 fragments when the oligonucleotide binding site for the F326 was used as a competitor (Fig. 3A, lane 3), indicating that the protein factor interacts with some other cloned nmDNA fragments. To further characterize the protein factor, we have partially purified the nuclear protein extract on a heparin–agarose column. The protein fraction containing F326 could be eluted in the range of 650–750 mM NaCl. It was separated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose filter, and hybridized with the radioactively labeled oligonucleotide binding site for F326. Only one protein band hybridized to the end-labeled F326 oligonucleotide binding site (Fig. 5). It corresponds to a protein with a molecular mass of 55 kDa.

To find out whether the F326 protein is present in other types of chicken cells, we have carried out a series of band-shift assays with the oligonucleotide binding site for F326 and different types of protein extracts, including those from actively proliferating cultured chicken erythroblasts (line HD3), fibroblasts, and the protein extract from the nuclear matrix prepared as described in Hofmann and Gasser [1991] (Fig. 3B). The F326 was found in the nuclear protein and the nuclear matrix extract from erythroblasts, and its concentration was greatly diminished in erythrocytes. The presence of the protein both in the nuclear matrix and the soluble protein fraction indicates that it may participate in attachment of DNA to the nuclear matrix.

nmDNA Fragment Containing the F326 Binding Site Specifically Binds to the Nuclear Matrix In Vitro

To study the role of the F326 protein in attachment of DNA to the nuclear matrix, we have used an in vitro MAR assay [Cockerill and Garrard, 1986]. The tested nmDNA fragment was end labeled and mixed with several end-labeled cloned DNA fragments, one of which contained a chicken MAR [Razin et al., 1991b] (positive control) and others that did not bind to the nuclear matrix (negative control). The mixture of DNA fragments was incubated with the nuclear matrix preparations in the presence of competitor DNA, as described in the Materials and Methods section. After incubation, the matrices were washed with several volumes of the incubation buffer to remove the entire nonbound probe, and the matrix-bound DNA was recovered by proteinase digestion of the matrix. The pattern of input and matrix-

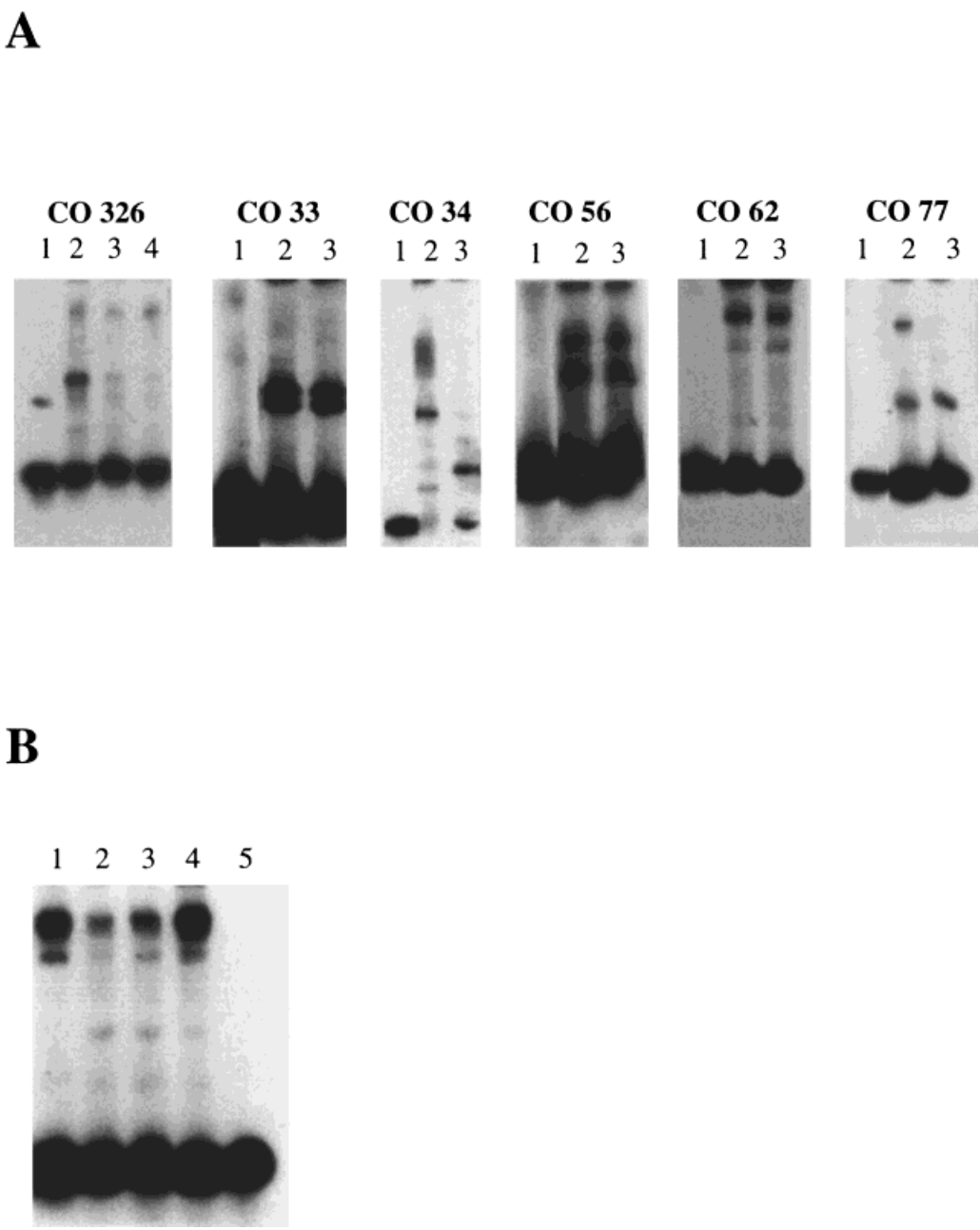

Fig. 3. **A:** Band-shift assay of complexes of different nuclear matrix-attached DNA (nmDNA) clones with nuclear protein extracts from proliferating cells (embryonic chicken fibroblast cells). Numbers indicate corresponding nmDNA clones. **Lane 1:** input DNA subfragment; **Lane 2:** incubation of an nmDNA fragment with purified nuclear DNA-binding protein extract from embryonic chicken fibroblasts (with a 50-fold excess of poly(dIdC); **Lanes 3 and 4:** the same as Lane 2, but a 25- and 50-fold molar excess of the oligonucleotide binding site for F326 was added as a competitor along with poly(dIdC). **B:** Band-shift assay of the oligonucleotide binding site for F326 protein with different nuclear protein extracts. **Lane 1:** incubation of the oligonucleotide binding site for F326 with the soluble nuclear protein extract from embryonic chicken fibroblasts (with a 50-fold excess of poly(dIdC); **Lane 2:** same as Lane 1, but incubated with the soluble nuclear protein extract from mature chicken erythrocytes; **Lane 3:** same as Lane 1, but incubated with the nuclear matrix extract from cultured chicken erythroblasts (line HD3); **Lane 4:** same as Lane 1, but incubated with the soluble nuclear protein extract from cultured chicken erythroblasts (line HD3); **Lane 5:** input oligonucleotide.

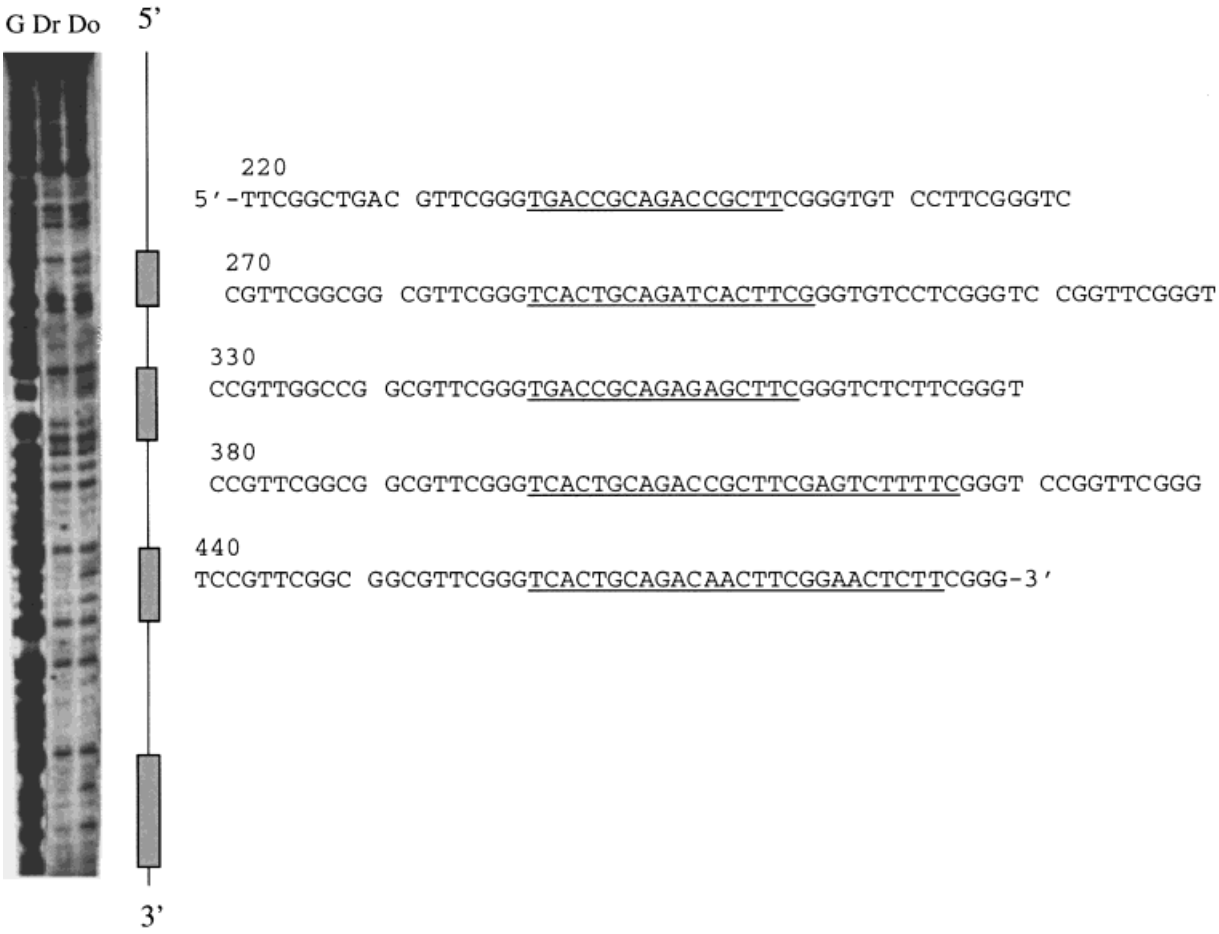

Fig. 4. Footprinting of a novel protein binding site in the cloned chicken erythrocyte nuclear matrix–attached DNA fragment. The end-labeled CO326 insertion was incubated with the protein extract from embryonic chicken fibroblasts and then briefly treated with DNaseI. Then the protein-associated fragments were separated from free DNA by preparative electrophoresis and applied to a sequencing gel. G, standard G cleavage pattern; Dr, DNaseI cleavage pattern of protein-bound fragment; Do, DNaseI cleavage pattern of the free fragment. Underlined nucleotides in the sequence (right) correspond to the protein-protected DNA areas.

bound labeled DNA was compared by electrophoresis.

Indeed, the CO326 fragment was preferentially associated with the nuclear matrix, whereas the other fragments, with the exception of the control MAR, were found in the supernatant only. To elucidate the role of the F326 protein in attachment of DNA to the nuclear matrix, we used its oligonucleotide binding site as a competitor. Addition of the 25-bp-long oligonucleotide to the incubation mixture nearly completely inhibited the attachment of the 490-bp-long CO326 fragment to the nuclear matrix (Fig. 6). The data obtained demonstrate that the studied fragment contains a matrix-associated region (MAR) and suggests the role of F326 protein in attachment of DNA to the nuclear matrix.

Plasmids Containing Nuclear Matrix–Associated DNA Can Promote Transient Replication in Cultured Chicken Erythrocyte Cells

Several lines of evidence exist on interconnection between the skeletal structures of the nucleus and DNA replication [for review, see Cook, 1991]. We tested whether the nmDNA fragments could promote replication of plasmids transiently transfected into chicken cultured erythroblast cells. All 10 nmDNA fragments, cloned into pTZ19, were cotransfected into cultured HD3 cells along with control plasmid (pTZ19). After 48 h, the cells were har-

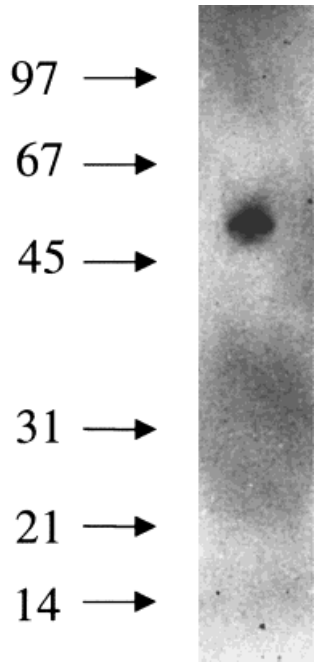

Fig. 5. Identification of a protein factor that interacts with the nuclear matrix–attached DNA fragments. Nuclear protein extract was partially purified on a heparin–agarose column. Protein fractions that interacted with the F326 oligonucleotide were pooled, separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and blotted onto a nitrocellulose filter. The blot was hybridized with the synthetic oligonucleotide probe containing the recognition site for F326.

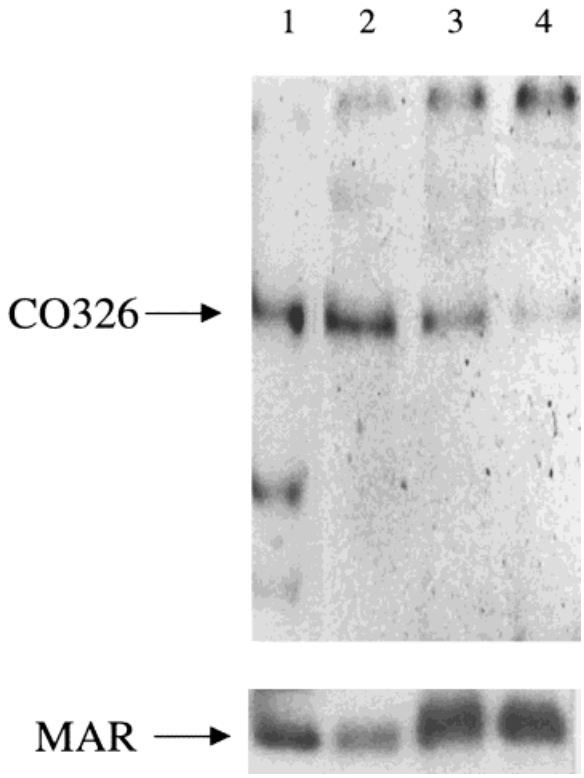

Fig. 6. Matrix-associated region (MAR) assay of the CO326 fragment. End-labeled CO326 fragment and a mixture of control end-labeled DNA fragments, one of which contained a chicken MAR [Razin et al., 1991b], were added to the nuclear matrices prepared along with the unlabeled competitor DNA as described in the Materials and Methods section, incubated at room temperature for 2 h, and washed with the incubation buffer. Matrix-bound DNA was purified by phenol extraction, electrophoretically separated in a polyacrylamide gel, and autoradiographed. **Lane 1:** 5% of the input DNA; **Lane 2:** incubation of the labeled DNA in the presence of sonicated salmon sperm DNA added as a competitor (0.25 mg/ml); **Lane 3:** same as Lane 2, but a 25-fold molar excess of the oligonucleotide binding site for F326 was added as a competitor along with the salmon sperm DNA; **Lane 4:** the same as Lane 2, but a 50-fold molar excess of the oligonucleotide binding site for F326 was added as a competitor along with the salmon sperm DNA. Arrows indicate the positions of the control MAR and the CO326 DNA.

vested, the plasmid DNA was isolated using the method of Hirt [1967], digested with the DpnI restriction endonuclease, and analyzed by transformation of *E. coli*.

The DpnI I restriction endonuclease only cuts DNA molecules that have been methylated on both DNA strands by the *E. coli Dam* methylation system. Hence, DNA that has replicated in eukaryotic cell is not accessible to this enzyme. DpnI digests only the input DNA that only persists in transfected cells >48 h. No replication of pTZ19 was detected in our experiments, whereas several nmDNA-containing plasmids became DpnI I-resistant, i.e., they replicated in cloned chicken erythroblast cells (Table I).

To check the nature of newly replicated DNA and exclude the possibility that it is produced in the result of integration-excision events [Holst et al., 1988], we have verified that there was no integration of plasmid DNA into chicken genome by genomic hybridization with the pTZ19 DNA used as a probe. No traces of integration were found (data not shown). We have also carried out a semiconservative replication assay [Landry and Zannis-Hadjopoulos, 1991]. After transfection of nmDNA-containing plasmids into the HD3 cells, the cells were incubated for 48 h in a medium containing the density-labeled precursor, bromodeoxyuridine. During this time, the cells underwent two cycles of replication. Plasmid DNA was isolated and applied to the neutral CsCl gradient to separate the DNA molecules containing density label in one or both strands from unlabeled

TABLE I. Properties of the Nuclear Matrix–Attached DNA (nmDNA) Fragments: Sequences With at Least 7/8 Homology to the FRC-1 Recognition Site Were Included

nmDNA insert	Binding to the nuclear matrix in vivo, % of the total plasmid DNA	Semiconservative replication	DpnI resistance, % of the total plasmid DNA	F236 recognition sites
pTZ19	7 ± 4	+/−	0.4 ± 0.2	0
CO30	2 ± 0.6	−	0.9 ± 0.3	0
CO326	45 ± 9	+	5.6 ± 1.1	5
CO33	ND	ND	ND	1
CO34	24 ± 12	+	2.5 ± 1.1	2
CO41	ND	−	0	0
CO56	6 ± 3	+/−	0.2 ± 0.1	2
CO62	7 ± 2	+/−	0.5 ± 0.3	0
CO65	12 ± 3	+/−	1.8 ± 0.8	1
CO73	15 ± 7	+/−	3.6 ± 1.3	2
CO77	22 ± 5	+	3.2 ± 0.9	1

ND, not done.

(i.e., nonreplicating DNA). The collected fractions were dot-blotted or separated by gel electrophoresis and hybridized to the specific nmDNA (or pTZ19) probe.

Results of the experiment are presented in Figure 7. Some of the nmDNA-containing plasmids underwent one or two rounds of replication in transfected cells. Interestingly, nmDNA-containing plasmids that proved to be replication positive in DpnI-sensitivity assay were also shown to replicate transiently in semiconservative assay (Table I).

nmDNA Fragments Enhance Attachment of Plasmid DNA to the Nuclear Matrix

Replication initiation in the plasmids studied was random and was not confined to the nmDNA inserts as monitored by two-dimensional electrophoresis in neutral agarose gels (data not shown), so we decided to check whether the enhancement in replication efficacy provided by the nmDNA fragments is due to their ability to direct the plasmids to the skeletal structures of the nucleus where the replication apparatus is located [for review, see Cook, 1991].

To check this, we transfected the nmDNA-containing plasmids into the HD3 cells, and after 48 h we isolated nuclear matrices and estimated the amount of plasmid DNA associated with the nuclear matrix for each of the studied nmDNA fragments. The results are presented in Table I. A correlation between the replication efficacy and association with the nuclear matrix is observed.

DISCUSSION

The mechanisms of DNA replication in cells of higher eukaryotes have become the subject of intensive study in many laboratories worldwide, yet the DNA sequences involved in initiation of DNA replication in cells of higher eukaryotes still have not been characterized in detail.

Our interest in DNA sequences located in the vicinity of the permanent attachment sites is mostly determined by the previous observation that replication origins coincide with or are located near the permanent attachment sites to the nuclear matrix [van der Velden et al., 1984b; Razin et al., 1986; Opstelten et al., 1989; Amati et al., 1990; Lagarkova et al., 1998].

Efficient replication of yeast DNA requires a minimal replication origin (ARS), a scaffold attachment region [Amati and Gasser, 1988; Amati et al., 1990], and an easily unwinding DNA element [Umek and Kowalski, 1988]. It is reasonable to suppose that replication origins in higher eukaryotes are also composed of several functional elements. Because the functionally active replication origins are located in the vicinity of nuclear matrix attachment sites, the study of DNA sequences originating from these attachment sites may allow the identification of the structures involved in the organization of

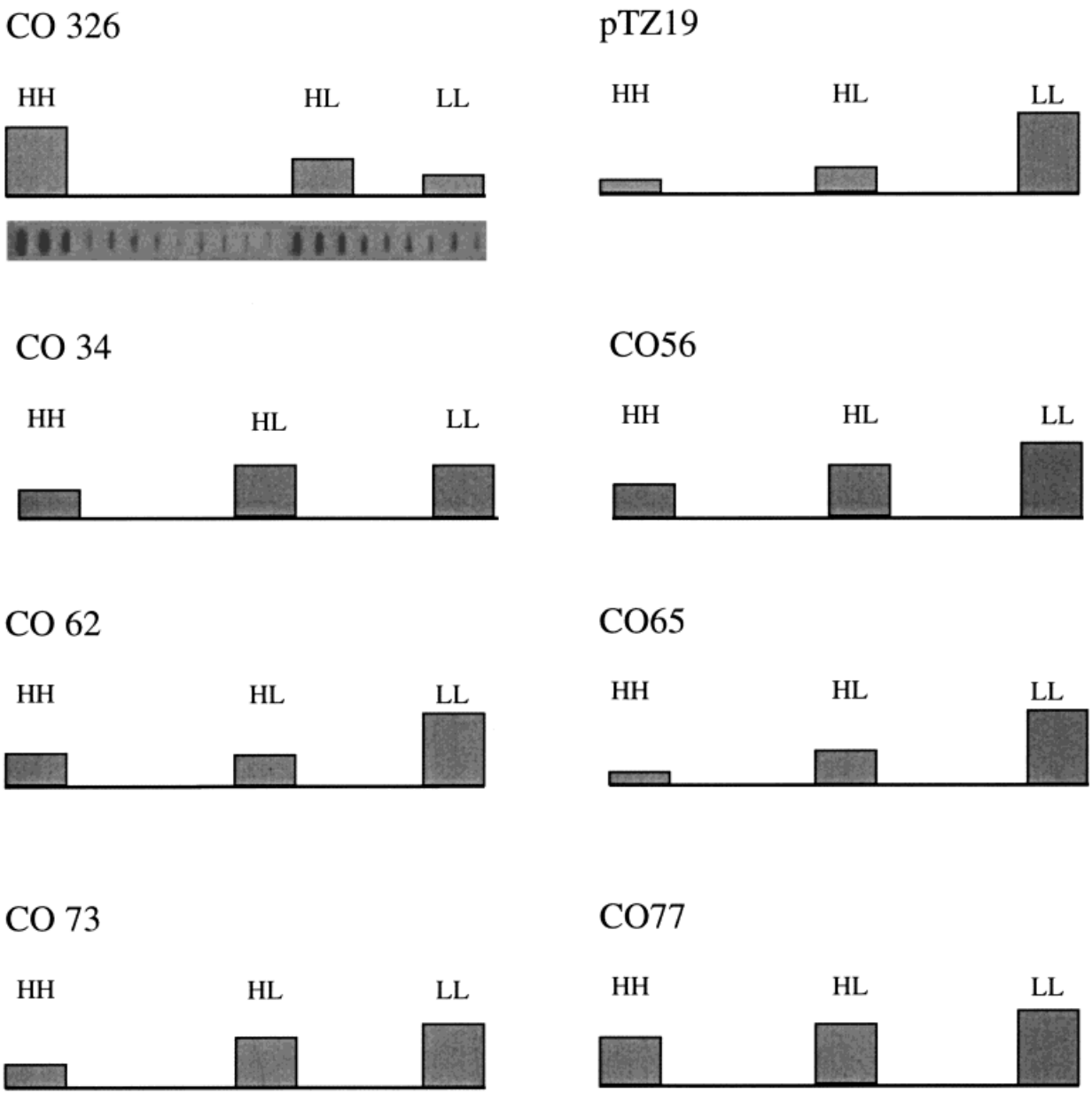

Fig. 7. Semiconservative transient replication assays with the nuclear matrix–attached DNA (nmDNA)-containing plasmids. The HD3 cells were transfected with different nmDNA-containing pTZ19 plasmids and then incubated in the growth medium containing 12.5 mg/ml bromodeoxyuridine for 24 and 48 h. Plasmid DNA was recovered, applied to a neutral CsCl gradient (initial refraction index 1.4150), and centrifuged at 65,000 rpm for 16 h. Fractions were collected starting from the bottom of the tubes, and the refractive index of every third fraction was measured. Aliquots of each fraction were dot blotted. The blots were probed with the corresponding radioactively labeled nmDNA fragments and were autoradiographed. The films were scanned using the Shimazu scanning densitometer. Positions of heavy–heavy, heavy–light, and light–light DNA bands are indicated correspondingly by letters HH, HL, LL.

a complete replication origin (including core element, regulatory, and auxiliary domains). We have sequenced 10 fragments of nmDNA that were overrepresented in a purified fraction of replication origins as compared to total DNA. Most of these DNA fragments shared a common consensus GCAGACCG/A, and had putative recognition sites for different sequence-specific protein factors and multiple internal imperfect repeats (data not shown). These factors are mostly involved in regulation of transcription, but there are reasons to believe that

at least some of them (e.g., NF-I and NF-III) are also involved in control of DNA replication [DePamphilis, 1988].

Our nmDNA sequences are relatively GC-rich, whereas most other authors have found AT-rich sequences to be preferentially attached to the nuclear matrix both in vivo and in vitro [Amati et al., 1990; Cockerill and Garrard, 1986; Opstelten et al., 1989; van der Velden et al., 1984a]. At the same time, GC-rich DNA stretches were observed in the sites of DNA attachment to the nuclear matrix in mouse 3T3 cells [Goldberg et al., 1983]. Recently, a GC-rich subset of MARs was described [Mielke et al., 1996]. Topoisomerase II, a major component of the nuclear matrix, preferentially interacts in vivo with GC-rich rather than with AT-rich sequences [Kas and Laemmli, 1992]. Moreover, in vivo topoisomerase II consensus [Kas and Laemmli, 1992] was found in 5 of 10 nmDNA fragments. This suggests that DNA topoisomerase II might play some role in attachment of DNA to the nuclear matrix.

We must also stress that our nmDNA fraction was isolated from inactive nuclei of chicken erythrocytes and may have a different composition from nmDNA found in normal proliferating cells. Differences in AT content in the nmDNA from cells in various proliferation states may reflect the difference in their functions in the cell.

We have also demonstrated that six nmDNA fragments interacted with sequence-specific DNA-binding proteins.

We have identified a novel binding site for a chicken DNA-binding protein. Interestingly, it contains the motif GCAGACCG/A, common to most of the studied fragments. In vitro band-shift experiments with the oligonucleotide binding site for the identified DNA-binding protein used as a competitor demonstrated that it interacted with other nmDNA fragments analyzed in the present study (Fig. 3A, lane 3).

This factor is present in several types of proliferating cells and is absent in resting ones (e.g., mature hepatocytes) and is also present in the nuclear matrix. This may imply that it has a role both in replication events and in attachment of DNA to the nuclear skeleton structures. The experiments show that a small oligonucleotide binding site for F326 protein can completely inhibit binding of the CO326 fragment to the nuclear matrix (Fig. 6), indicating that the F326 protein plays some role in the interaction of DNA with the nuclear matrix.

The F326 protein seems to be distributed between the nuclear matrix and the soluble fraction of the nucleus. Similar distribution pattern is observed in most proteins that constitute the nuclear matrix, e.g., DNA topoisomerase II that is present in the nucleus both in the soluble and the scaffold-associated states. This property may in fact aid the anchoring of DNA sequences, because the F326 protein may attach to DNA and guide it to the nuclear matrix.

Interestingly, the CO326 fragment contains five copies of a 60-bp-long direct repeat with the F326 recognition site. We have tested the chicken genome for the presence of this repeat and found out that it is represented in the genome by ~30 copies (data not shown). Recently, we have isolated several genomic DNA clones containing this repeat. They are being characterized now in our laboratory.

The size and composition of a minimal replication origin in higher eukaryotes is not known yet. Moreover, the replication origins may contain a set of functional elements (e.g., easily unwinding DNA, direct and/or inverted repeats, bent DNA, matrix attachment sites) rather than strict homologies. This may explain the fact that we did not find a pronounced homology between the sequenced nmDNA fragments and the relatively low efficiency of autonomous replication of nmDNA-containing plasmids.

Dramatic advances have recently been made in studies of yeast replication origins because of discovery of the so-called autonomously replicating sequences. Several attempts to obtain long-term autonomous replication of plasmids in mammalian cells [Ariga et al., 1987; Holst et al., 1988; McWhinney and Leffak, 1990; Mielke et al., 1996] have produced controversial results.

In our analysis of replication origin elements, we used a short-term transient replication assay that has been developed by a number of laboratories [Heinzel et al., 1991; Landry and Zannis-Hadjopoulos, 1991; Masukata et al., 1993]. Some of the tested nmDNA-containing plasmids were able to replicate in actively proliferating cultured chicken erythroblast cells to a higher extent than the vector DNA alone (Fig. 7) and randomly chosen short

DNA fragments of different origin (data not shown). Autonomous replication was observed during at least two cell cycles. The observed transient autonomous replication was never very efficient. This is in agreement with the data of Heinzel et al. [1991], who found that replication efficiency of plasmids introduced into human cells depends on the size and source of DNA. In our case, very small (200–500 bp) fragments of chicken DNA were used for the transient replication assay. The CO326 fragment, which consists of five copies of a repeat, has the highest replication efficacy. Krysan et al. [1993] observed higher replication efficacy when multimeric human DNA was used for the autonomous replication assays.

We have also shown that the nmDNA sequences enhance attachment of plasmid DNA to the nuclear matrix, and this enhancement in general correlates with the replication efficacy of the studied constructs (Table I, Fig. 7). These results are in agreement with the observations of Cossons et al. [1997].

We conclude that nuclear matrix attachment may enhance replication initiation events in the cells of higher eukaryotes. Further studies and development of more reliable methods to detect initiation of replication in higher eukaryotes will help us to understand this mechanism in more detail.

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