Journal of Cellular Biochemistry 104:773–784 (2008)

Fine Mapping and Functional Activity of the Adenosine Deaminase Origin in Murine Embryonic Fibroblasts

Sahar Sibani, Emmanouil Rampakakis, Domenic Di Paola, and Maria Zannis-Hadjopoulos*

McGill Cancer Center and Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3G 1Y6

Abstract DNA replication initiates at origins within the genome. The late-firing murine adenosine deaminase (mAdA) origin is located within a 2 kb fragment of DNA, making it difficult to examine by realtime technology. In this study, fine mapping of the mAdA region by measuring the abundance of nascent strand DNA identified two origins, mAdA-1 and mAdA-C, located 397 bp apart from each other. Both origins conferred autonomous replication to plasmids transfected in murine embryonic fibroblasts (MEFs), and exhibited similar activities in vivo and in vitro. Furthermore, both were able to recruit the DNA replication initiator proteins Cdc6 and Ku in vitro, similar to other bona fide replication origins. When tested in a murine Ku80$^{-/-}$ cell line, both origins exhibited replication activities comparable to those observed in wildtype cells, as did the hypoxanthine-guanine phosphoribosyltransferase (HPRT) and c-*myc* origins. This contrasts with previously published studies using Ku80-deficient human cells lines and suggests differences in the mechanism of initiation of DNA replication between the murine and human systems. J. Cell. Biochem. 104: 773–784, 2008.

Key words: initiation of DNA replication; replication origin; mouse; adenosine deaminase; Ku80; nascent DNA; realtime PCR

Initiation of DNA replication is orchestrated by the sequential assembly of protein complexes at origins of replication. Numerous origins have been isolated and characterized in several model systems, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, Chinese hamster ovary cells (CHO), murine and human cells (reviewed in [Todorovic et al., 1999, 2005]). In the majority of cases, the origin-containing regions spanned only a few hundred base pairs, making them relatively easy to study. The most notable exception is the Chinese hamster dihydrofolate reductase (DHFR) locus, in which numerous origins were detected within a 50 kb region [Dijkwel et al., 2002]. The most detailed mapping has recently been pioneered using genome tiling oligonucleotide microarrays of human chromosomes 21 and 22, generating a wealth of information regarding the location and time of activation of these origins [Jeon et al., 2005].

Murine origins have been described in the adenosine deaminase (AdA) [Carroll et al., 1993; Virta-Pearlman et al., 1993], immunoglobulin heavy chain enhancer [Ariizumi et al., 1993; Iguchi-Ariga et al., 1993], immunoglobulin kappa light chain genes [Hatton and Schildkraut, 1990], hypoxanthine-guanine phosphoribosyltransferase (HPRT) [Cohen et al., 2004], c-*myc* promoter [Ariga et al., 1989; Sudo et al., 1990; Girard-Reydet et al., 2004], and ribosomal DNA [Berger et al., 1997; Grozdanov et al., 2003] loci. Of these, the AdA origin was the best mapped, identifying an origin of bidirectional replication (OBR) within a 2 kb region. Nevertheless, such a fragment is prohibitively large for contemporary technological applications such as realtime PCR, RIP mapping, and chromatin immunoprecipitation (ChIP) experiments, necessitating the fine-mapping of this origin with higher resolution.

Once origins are fine-mapped, their binding proteins can be identified and their role in DNA

Sahar Sibani and Emmanouil Rampakakis contributed equally to this work.

Grant sponsor: CIHR (M.Z-H.).

*Correspondence to: Maria Zannis-Hadjopoulos, PhD, 3655 Promenade Sir William Osler, Rm. 710, Montreal, QC H3G 1Y6, Canada. E-mail: maria.zannis@mcgill.ca

Received 15 September 2007; Accepted 14 November 2007

DOI 10.1002/jcb.21662

replication analyzed. One protein recently identified to promote initiation of DNA replication in human cells is the heterodimeric Ku protein (Ku70/Ku80), purified from HeLa cells through its ability to bind to the monkey replication origin *Ors*8 [Ruiz et al., 1999]. It was found to complex with several replication proteins, including Orc2, DNA polymerases α, δ, and ε, PCNA, RF-C, and topoisomerase II [Matheos et al., 2002]. Its in vivo binding to the human lamin B2, β-globin, and c-*myc* origins [Sibani et al., 2005a], hamster DHFR origin, and monkey *Ors*8 and *Ors*12 origins was shown to be sequence- and cell cycle-specific, peaking at late G_1 and decreasing thereafter [Novac et al., 2001]. Recent work using HCT116 $Ku80^{+/-}$ haploinsufficient cells revealed that Ku may be involved in the loading and/or stabilization of the Orc-3, -4, and -6 subunits to Orc-2 at origins [Sibani et al., 2005b]. Similar findings were reported in in vitro studies of purified origin-binding protein complexes from *S. cerevisiae*, where Ku stabilized the interaction of the ORC complex with *ARS*121, a yeast replication origin [Feldmann et al., 1996]. Furthermore, Ku80 deficiency in human HCT116 $Ku80^{+/-}$ cells led to a 2 h delay in their entry into S-phase and a fourfold decrease in origin activity [Sibani et al., 2005a]. In human cells, Ku is necessary for cellular viability, as HCT116 $Ku80^{-/-}$ die by apoptosis following 8–10 doublings [Li et al., 2002]. In contrast, primary murine $Ku80^{-/-}$ embryonic fibroblasts are viable albeit displaying a prolonged doubling time of 41 h relative to 23 h in wildtype cells (23).

In this study, we have undertaken a fine mapping analysis of the murine AdA origin, and identified two origins of approximately 200 bp each. Their in vivo replication activities in $Ku80^{+/+}$ and $Ku80^{-/-}$ MEF cells were found to be comparable, while both cell lines were able to replicate the murine AdA origin episomally with similar efficiencies, indicating no effect of Ku80-deficiency on DNA replication.

MATERIALS AND METHODS

Cell Lines

$Ku80^{-/-}$ MEF cells immortalized by SV40 large T-antigen (a generous gift from Dr. Andre Nussenzweig) were generated by homologous recombination and cre-loxP technology, as described in [Nussenzweig et al., 1996]. The knockout and wildtype parental cells were cultured in high glucose DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin (hereafter referred to as complete medium), and maintained at 37°C, 5% CO_2.

Isolation and Quantification of Nascent Strand DNA

Isolation of nascent DNA was done exactly as described previously [Sibani et al., 2005a,b]. DNA fragments 0.5–1 kb in size were isolated, resuspended in 200 μl dH_2O and quantified by realtime PCR. The SYBR Green JumpStart Taq ReadyMix for Quantitative PCR kit (Sigma) was used to quantify DNA, as per the manufacturer's instructions, in the LightCycler instrument (Roche Diagnostics, Basel, Switzerland). The DNA abundance for each amplicon is reported as fold difference relative to the amplicon with the lowest activity. The sequences and annealing conditions of all primer sets used are available upon request.

Nuclear Extract Preparation and Quantification

Nuclear extracts (NEs) were prepared according to the Dignam protocol [Dignam et al., 1983] and the protein concentration of each extract preparation was determined using the Bradford protein assay (BioRad, Hercules, CA).

Electrophoretic Mobility-Shift Assay (EMSA)

Nuclear cell extracts (10 μg) were incubated with 0.4 fmoles of ^{32}P-labeled PCR products corresponding to fragments mAdA-1, -C, or -D regions (Fig. 1) and the origin related to the HPRT locus [Cohen et al., 2004], for 1 h on ice. Binding to oligonucleotides was performed in the presence of 2 μg poly dI-dC (Amersham-Pharmacia), used as non-specific competitor, and in a final volume of 20 μl including binding buffer (10 mM Tris-HCl, pH 7.5, 80 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X-100, 4% glycerol). The mixtures were subjected to electrophoresis on a native 5% PAGE at 160 V in 0.5 × TBE and the gels were then dried and subjected to autoradiography. For competitive EMSA assays, increasing molar excess amounts of cold probe were included in the reactions, as indicated in the figures. For the interference EMSA experiments, either 1 μg of anti-Ku80 (M-20) or 6 μg anti-Cdc6 antibody (180.2, Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit serum were added to the EMSA reaction.

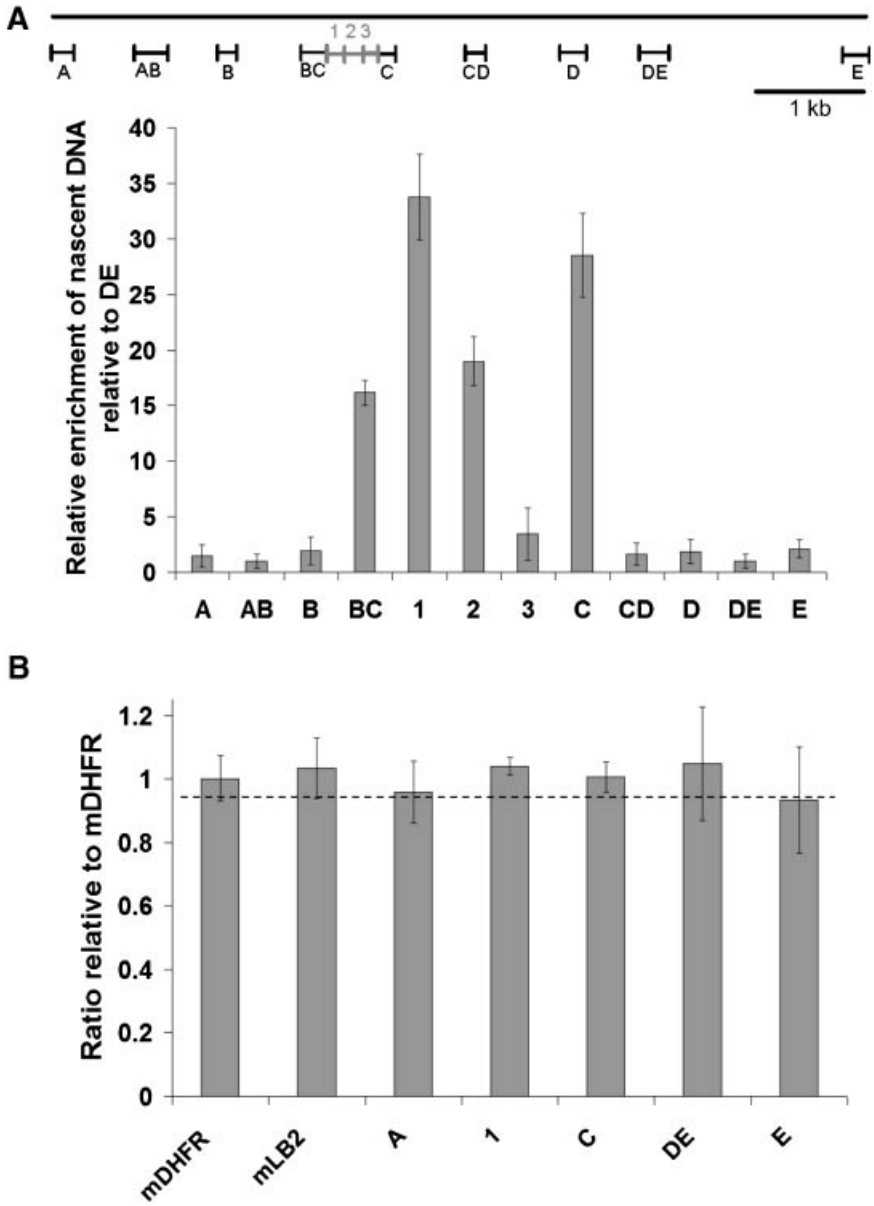

Fig. 1. Fine mapping of the late-firing mAdA locus. **A**: **Top panel** Schematic representation of the mAdA locus, indicating (in black) the locations of the amplicons examined in the original study (Virta-Pearlman et al., 1993)(A, B, C, D, and E) along with the four intervening amplicons added in this study (AB, BC, CD, and DE). Amplicons 1, 2, and 3 (in gray) were introduced for further fine mapping of the region between BC and C. **Bottom panel**: Abundance of nascent strand DNA for each of the mAdA regions is reported as fold enrichment relative to region mAdA-DE, which was set to 1. Abundance of mAdA-DE was 4.8×10^4 nascent DNA molecules per 20 μg genomic DNA. The average and standard deviation of at least four experiments is shown. **B**: The abundance of five fragments (A, 1, C, DE, E) in the mAdA locus was determined in genomic DNA isolated from serum-starved cells. The abundance of each region is expressed as a ratio relative to single copy DHFR uniSTS. Another uniSTS, LB2 is also included for comparison. Results are averages of three experiments and their standard deviations. The dotted line represents a ratio of one, that is, a single copy DNA in a haploid genome.

Western Blot Analysis Quantification

Western blot analysis was carried out according to standard protocols [Sambrook et al., 1989]. Briefly, the indicated amounts of NEs were resuspended in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 min and loaded on a 8% SDS–PAGE gel. Following electrophoresis, the proteins were transferred onto a PVDF membrane and the membrane was immunoblotted with a

1/100 dilution of anti-Ku80 (M-20, Santa Cruz Biotechnology, Santa Cruz, CA) or a 1/1,000 dilution of anti-actin (Sigma) antibody for 1 h at room temperature. The corresponding HRP-conjugated secondary antibodies were added and the protein bands were visualized by chemiluminescence according to the manufacturer's instructions (Perkin-Elmer, Woodbridge, ON). Blocking of the anti-Ku80 antibody (M-20) was performed by incubation with sevenfold mass excess of the Ku80 blocking peptide (sc-1485P) for 2 h at 37°C and 24 h at 4°C.

Plasmids and Episomal DNA Replication Assay

PCR products of the mAdA-1, -C, and -D fragments (Fig. 1) were obtained and cloned into pBlueScript II (pBS) (Stratagene, La Jolla, CA), hereafter referred to as mAdA vectors. Mini-preparations of the plasmid DNA were made using Qiagen's Qiaprep Spin Miniprep kit, according to manufacturer's instructions (Qiagen, Mississauga, ON). All plasmids were sequenced to check for lack of PCR-introduced mutations.

The episomal DNA replication assay based on *Dpn*I resistance was performed as previously described [Landry and Zannis-Hadjopoulos, 1991; Nielsen et al., 1994]. Transfection efficiency was corrected for by co-transfection with 1 μg of pM1-SEAP (secreted human placental alkaline phosphatase) vector (Roche Molecular Biochemicals, Indianapolis, IN), and assaying for the alkaline phosphatase activity using the SEAP reporter gene assay (Roche Applied Sciences), as per the manufacturer's instructions.

RESULTS

Previous mapping of the late-firing murine adenosine deaminase (mAdA) replication origin inferred the existence of an origin between regions mAdA-B and -C (Fig. 1A, top panel) [Virta-Pearlman et al., 1993]. However, this area of over 2 kb is too large to analyze by contemporary real-time technology. In order to refine the mapping of the mAdA origin, four intervening amplicons were added (mAdA-AB, -BC, -CD, and -DE) to the five amplicons described in the original study (mAdA-A, -B, -C, -D, and -E). Preliminary data indicated that an origin might reside between mAdA-BC and -C, a region of approximately 870 nucleotides (data not shown), whereupon an additional three amplicons (mAdA-1, -2, and -3) were introduced to that region to further refine the mapping. The origin activity over that region was quantitatively analyzed by real-time PCR and the pattern of nascent strand DNA abundance revealed two active origins, mAdA-1 and mAdA-C, separated by 397 bp (Fig. 1A, lower panel). mAdA-1 exhibits approximately a 34-fold higher activity than the region with the least activity, mAdA-DE (Fig. 1A, lower panel). Flanking mAdA-1 are two shoulders, mAdA-BC and mAdA-2, that have a 9- and 10-fold higher abundance than mAdA-DE, respectively (Fig. 1A, lower panel), an indication of bidirectional replication. The second origin is located at amplicon mAdA-C, as described in the original study [Virta-Pearlman et al., 1993], with approximately 29-fold higher activity than region DE (Fig. 1A, lower panel). The shoulders of this origin have a much lower abundance than those of origin mAdA-1 with the adjoining amplicons mAdA-3 and mAdA-CD present at 3.5- and 2.5-fold greater abundance than mAdA-DE, respectively (Fig. 1A, lower panel).

Some studies have identified amplification in the mAdA locus and its surrounding regions in certain cell lines [Carroll et al., 1993]. To verify that the data obtained from the nascent DNA abundance analysis were not due to aberrant amplification in the genomic DNA or the presence of homologous sequences within other regions of the genome, the copy numbers of five amplicons spanning the entire mAdA region (mAdA-A, -1, -C, -DE, and -E) were measured (Fig. 1B). The abundance of these regions in genomic DNA isolated from serum-starved, non-replicating cells relative to two single-copy unique sequence tagged sites (uniSTSs) obtained from NCBI located within the murine DHFR and lamin B2 loci was measured. The results (Fig. 1B) indicated that the entire 9.3 kb region examined was single copy relative to the DHFR and lamin B2 loci, indicating that it is present as single copy per haploid genome and validating the nascent DNA data.

Several origin binding proteins have been reported to interact with origin-containing DNA sequences, including Ku and Cdc6 (reviewed in [Bell and Dutta, 2002; Zannis-Hadjopoulos et al., 2004; Sibani et al., 2005a,b]). To test whether the newly mapped origins in the mAdA locus could recruit origin-binding proteins in vitro, EMSA were carried out (Fig. 2). NEs from wildtype MEF cells were able to bind

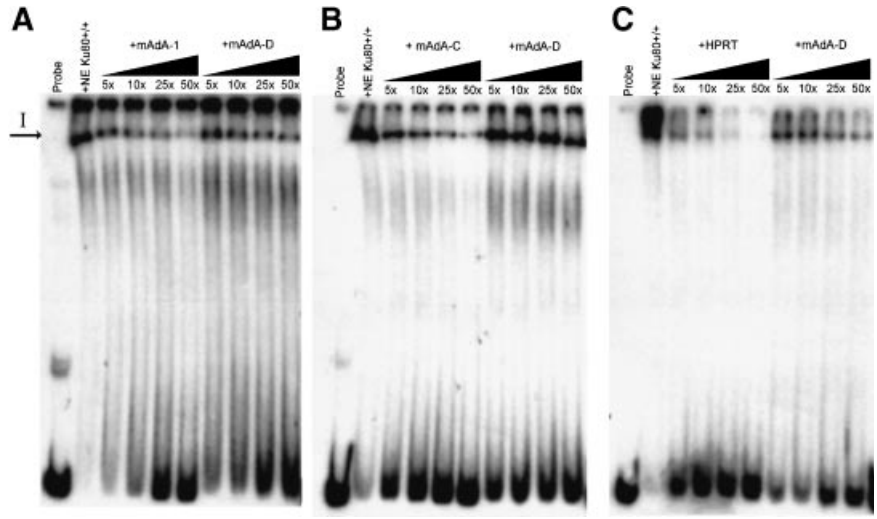

Fig. 2. In vitro protein-origin DNA complex formation. Electromobility shift assays were performed using NEs from wildtype MEF cells and the mAdA-1 (**A**), mAdA-C (**B**), or HPRT (**C**) origins as probes (the protein–DNA complex is indicated by arrow I). For each origin, the protein DNA complex (**lane 2**) was competed with increasing concentrations of either unlabeled probe DNA (**lanes 3–6**) or non-origin containing mAdA-D (**lanes 7–10**). The molar excess of the competitor relative to the probe is indicated across the top.

and shift the mAdA-1 (Fig. 2A, arrow I), mAdA-C (Fig. 2B, arrow I), and HPRT (Fig. 2C, arrow I) origins. The shifts were of relatively equivalent magnitude, suggesting that similar protein complexes bind to each origin. Dissociation of the protein-origin complex in the presence of origin-containing DNA, but not in the presence of non-origin DNA (mAdA-D) indicated a sequence-specific association for all three origins (Fig. 2).

To analyze whether the protein complex(es) assembled on the mAdA-1, mAdA-C, and HPRT sequences contained DNA replication proteins, EMSA interference assays were employed where an antibody targeting Cdc6 was added to the EMSA reaction to inhibit Cdc6 interaction with the DNA (Fig. 3). Cdc6 is a well-studied initiator protein of DNA replication, required for origin licensing by promoting stable loading of the MCM2-7 complex at the origins [Randell et al., 2006]. Addition of the anti-Cdc6 antibody decreased the molecular weight of the complex, leading to formation of a smaller complex of the mAdA-1 (Fig. 3A, arrow II), mAdA-C (Fig. 3B, arrow II), and HPRT origins (Fig. 3C, arrow II). In contrast, when the antibody was added to the origin-lacking mAdA-D–protein complex, no change in the bandshift pattern was observed, indicating the absence of Cdc6 from that protein complex, as expected (Fig. 3D, arrow II).

Ku is involved in multiple DNA maintenance processes, including DNA repair, recombination, telomere maintenance, and DNA replication in monkey and human cells [Downs and Jackson, 2004; Egel, 2004; Zannis-Hadjopoulos et al., 2004; Fisher and Zakian, 2005; Lombard et al., 2005]. To test if Ku80 bound the newly identified origins in the AdA locus, another interference EMSA was employed using an antibody that targets the C-terminus of the murine Ku80 and NEs from MEFs. The antibody prevented protein–DNA complex formation for the mAdA-1 (Fig. 4A, lane 4, arrow I), mAdA-C- (Fig. 4B, lane 4, arrow I), and HPRT origins (Fig. 4C, lane 4, arrow I) indicating that Ku80 is required for protein complex formation on those origins. Yet when the antibody was added to the mAdA-D–protein complex (Fig. 4D, lane 4, arrow I) no such dissociation was observed indicating that the C-terminal domain of Ku80 is not involved in end binding. In light of Ku's role as a DNA-end binding protein, the ability of this C-terminal targeted antibody to dissociate Ku80 from the origin-containing mAdA-1, -C, and HPRT probes, but not the mAdA-D probe, suggests that the C-terminus of the murine Ku80 may be the region of the protein involved in DNA origin binding. When another antibody targeting the central heterodimeric region of Ku was used, no protein–DNA dissociation was seen in the

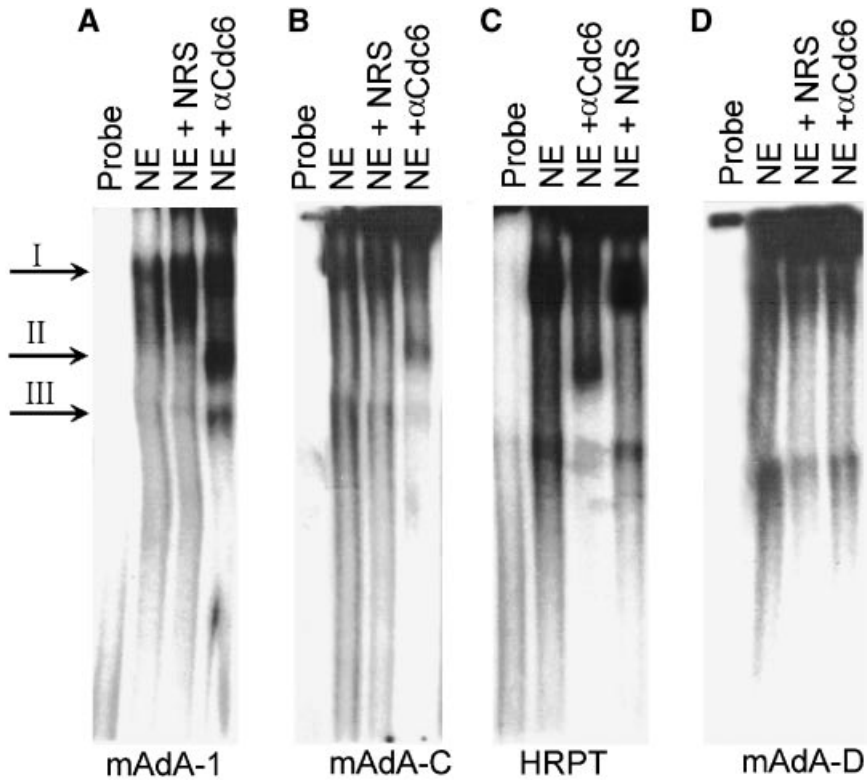

Fig. 3. In vitro Cdc6 association with mAdA-1 and mAdA-C origins. NEs were incubated with either mAdA-1(**A**), mAdA-C (**B**), or HPRT (**C**) origins DNA (**lane 2**, indicated by arrow I), and competed with anti-Cdc6 antibody resulting in a smaller complex (arrow II). The anti-Cdc6 antibody did not affect mAdA-D–protein complex (**D**, **lane 4**) indicating the absence of Cdc6 in the complex. Normal rabbit serum is used as a negative control for all three probes (**lane 3**). A protein degradation product can also be seen (arrow III).

EMSAs with the HPRT origin (data not shown), further corroborating the involvement of the C-terminus of Ku80 in origin binding. Finally, to test the specificity of the dissociating antibody, a blocking peptide was used to neutralize the antibody followed by Western blot analysis of wildtype and $Ku80^{-/-}$ NEs (Fig. 5). In the presence of the blocking peptide no signal for Ku80 was obtained indicating that the antibody is specific in its epitope binding.

In simple eukaryotes, the *cis*-acting elements that promote DNA replication coincide with the location of unwinding and DNA synthesis, while in higher eukaryotes, these two elements may not coincide. To determine whether the amplicons identified as origins (mAdA-1 and mAdA-C) can support DNA replication, their ability to confer autonomous replication on bacterial plasmids lacking a mammalian origin was examined in wildtype MEF cells, using the transient episomal replication assay, as previously described [Landry and Zannis-Hadjopoulos, 1991; Nielsen et al., 1994]. A plasmid containing the human lamin B2 origin (LB2P) was used as a positive control, while the parental vector, pBS, was used as negative control. The replication capacity of each amplicon was assessed by *Dpn*I resistance [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Nielsen et al., 1994] and expressed relative to that of LB2P (Fig. 6, white bars). Both mAdA-1 and -C were able to confer autonomous DNA replication on the bacterial plasmid with similar efficiencies as LB2P. Relative to LB2P, mAdA-1, and -C replicated with approximately 91 and 83% efficiency in wildtype MEF cells, respectively. In contrast, no episomal replication activity was detected for the origin-lacking mAdA-D region nor the pBS backbone vector. These data indicated that amplicons mAdA-1 and mAdA-C contained the sequences required to support autonomous DNA replication.

Since Ku80-deficiency in humans decreased origin firing [Sibani et al., 2005a], we examined the effect of the absence of murine Ku80 on the

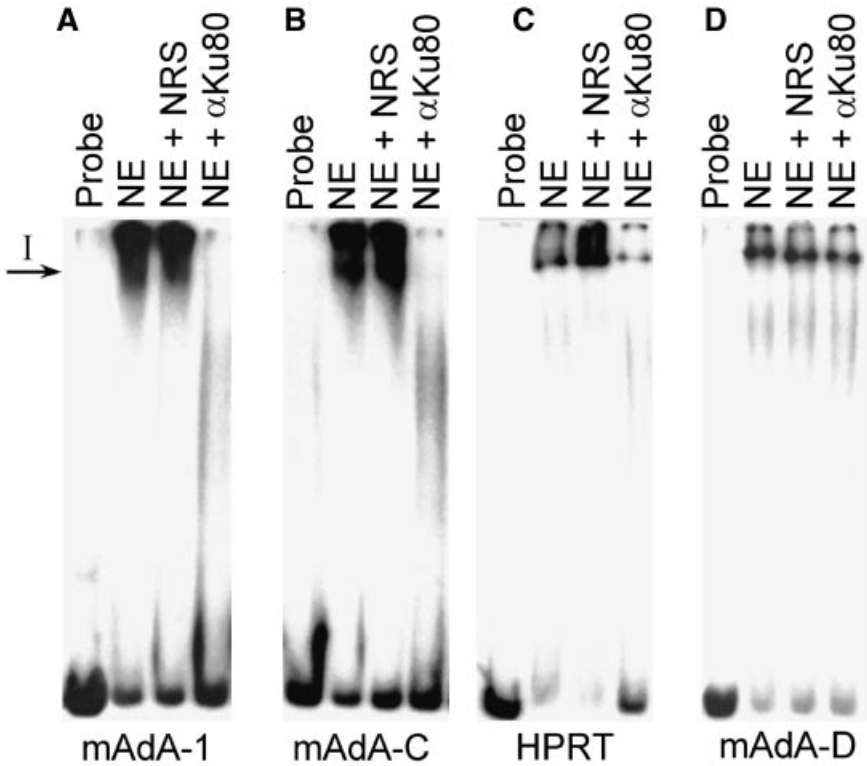

Fig. 4. In vitro association of Ku80 with mAdA-1 and mAdA-C origins. Anti-Ku80 antibody was incubated with NEs and added to origin DNA. The protein complexes previously seen for the mAdA-1 (**A**), mAdA-C (**B**), and HPRT (**C**) origins (**lane 2**, arrow I) are inhibited in the presence of the antibody (**lane 4**, arrow I). This effect is not seen in the origin-lacking mAdA-D DNA (**D**). Normal rabbit serum is used as a negative control (**lane 3**).

activation of the mAdA origins characterized in this study. Two methods were employed to ascertain DNA replication activity in Ku80$^{-/-}$ MEF: the transient episomal DNA replication assay and in situ nascent strand DNA abundance. Transfection of plasmids harboring either of the two origins, mAdA-1 or mAdA-C, showed that they were both able to replicate episomally in Ku80$^{-/-}$ MEFs, with similar efficiencies to that of the LB2P-plasmid (Fig. 6, gray bars). Similarly, the abundance of nascent strand DNA for mAdA-1 in Ku80$^{-/-}$ cells was 94% of that in Ku80$^{+/+}$ cells (16.9- vs. 17.9-fold enrichment of mAdA-1 relative to mAdA-E for Ku80$^{-/-}$ and Ku80$^{+/+}$, respectively) (Fig. 7A). Likewise, HPRT-P and Myc-P showed no

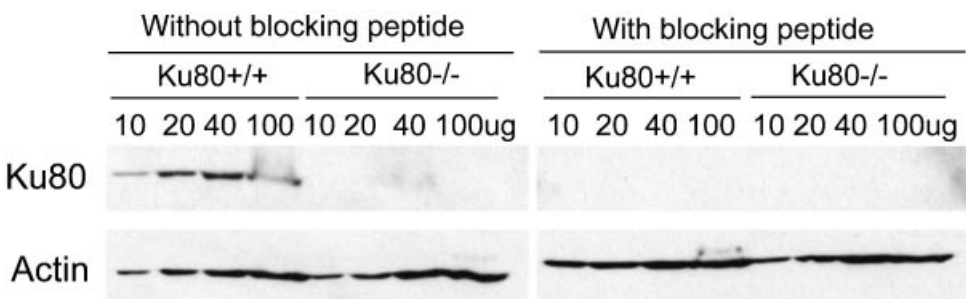

Fig. 5. Specificity of the anti-Ku80 antibody. The specificity of the anti-Ku80 antibody used in Figure 4 was tested by two methods. First, the absence of a reactive protein in the Ku80$^{-/-}$ NEs indicates that the antibody does not interact with other proteins (**left panel**, **lanes 5–8**). Second, the absence of any signals upon the use of a blocking peptide prior to immunoblotting indicates that the antibody was specific to Ku80 (**right panel**, **lanes 1–4**). Actin was used as a loading control.

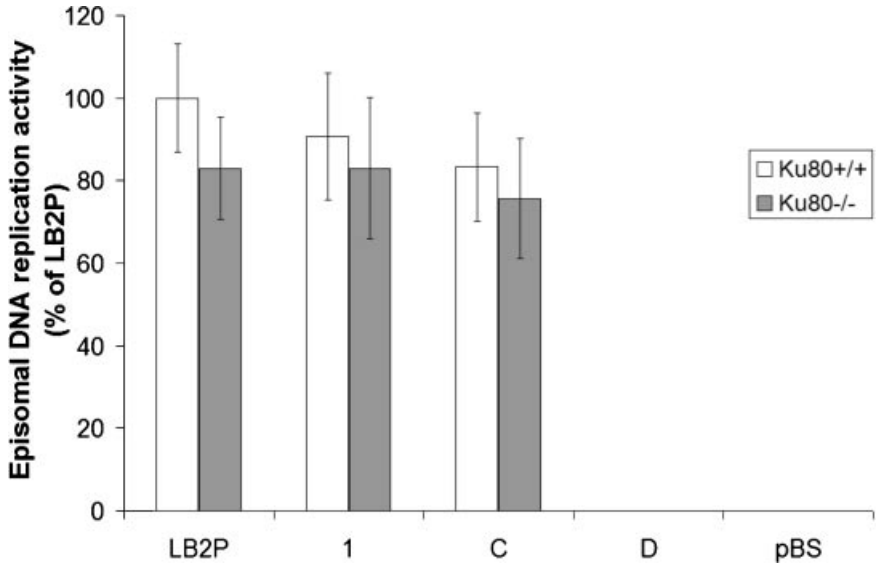

Fig. 6. Episomal DNA replication capacity of the mAdA origins. The human lamin B2 origin (LB2P) or various fragments of the mAdA locus (mAdA-1, -C, -D) were cloned into bacterial plasmid, pBS, and transfected into wildtype (white bars) or Ku80$^{-/-}$ MEF cells (gray bars). Their autonomous replication capacity was assayed using the *DpnI* resistance assay 72 h following transfection (Landry and Zannis-Hadjopoulos, 1991; Nielsen et al., 1994). The data represent the average and standard deviations of three independent experiments.

significant change in their activity in Ku80$^{-/-}$ cells relative to wildtype Ku80$^{+/+}$ MEFs (7.5- vs. 7.9-fold enrichment of HPRT-P relative to mAdA-E for Ku80$^{-/-}$ and Ku80$^{+/+}$ cells, respectively, and 16.1- vs. 15.4-fold enrichment of Myc-P relative to mAdA-E for Ku80$^{-/-}$ and Ku80$^{+/+}$ cells, respectively, Fig. 7B and C). The abundance of the negative control region, mAdA-E, was equally low in both cell lines (0.99-fold abundance of mAdA-E in Ku80$^{-/-}$ relative to Ku80$^{+/+}$ cells) (Fig. 7A). Interestingly, the c-*myc* origin was almost as active as the mAdA origin, while the HPRT origin displayed a twofold lower activity (17.9-, 7.9-, and 15.4-fold enrichment of mAdA-1, HPRT-P, and Myc-P relative to mAdA-E, respectively). Thus, Ku80 deficiency did not result in a significant decrease in the transient episomal replication of plasmids harboring the mAdA origins, nor their in situ chromosomal replication.

DISCUSSION

Quantification of nascent strand DNA at the mAdA locus revealed two origins spaced 397 bp apart with similar strengths and frequency of use. The presence of two replication origins at the mAdA locus in such close proximity to each other is reminiscent of other mapped eukaryotic origins, including the human c-*myc* [Tao et al., 2000] and β-globin origins [Kamath and Leffak, 2001] as well as the multiple origins comprised in the Chinese hamster DHFR locus [Dijkwel et al., 2002]. Redundancy of replication origins within a locus might represent a fail-safe mechanism that ensures initiation of DNA replication within that region. Thus, if one origin fails to become activated or is mutated, another is present to take its place ensuring genomic stability and continued propagation of the cell.

The close proximity of the two origins makes it unlikely that both would fire within any individual cell, especially since the MCMs have been reported to bind regions up to 3 kb surrounding an origin [Edwards et al., 2002]. Use of the more elaborate molecular combing method [Herrick and Bensimon, 1999; Lebofsky and Bensimon, 2003; Debatisse et al., 2004] should produce definitive data for the determination of intra-strand origin firing. Transient episomal DNA replication assays indicated that the mAdA-1 and mAdA-C amplicons were both able to confer replication autonomy onto a bacterial plasmid lacking a mammalian origin. Both origins again had similar strengths in firing, and were very comparable to the strong human lamin B2 origin (Fig. 6), but the

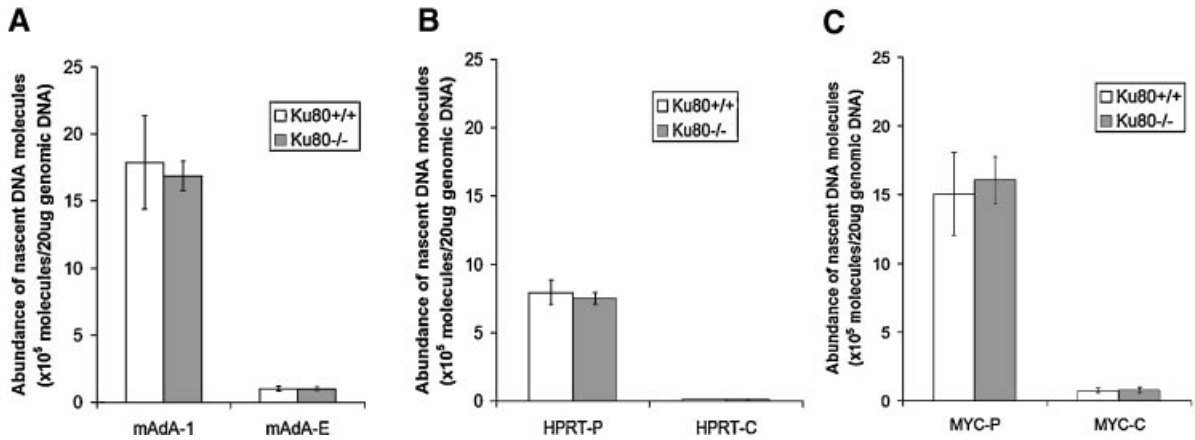

Fig. 7. Activities of the mAdA-1, HPRT, and Myc origins in situ in Ku80$^{-/-}$ and Ku80$^{+/+}$ MEF cells. Nascent strand DNA was prepared and assayed for the abundance of mAdA-1 and -E (**A**), HPRT-P and -C (**B**), and Myc-P and -C (**C**), in Ku80$^{+/+}$ and Ku80$^{-/-}$ MEF cells. For the HPRT and c-*myc* origins, -P designates the origin while -C represent a region lacking origin activity. The average and standard deviations of three experiments are shown.

shoulders of mAdA-C origin were of lower abundance relative to those of mAdA-I. There are two possible explanations for this observation: the first is that mAdA-C origin is fired less frequently than the mAdA-I origin, hence producing lower shoulders. However, the data presented in Figure 1 indicate that both origins have similar peak values, suggesting that differential origin activity is not the case. A more plausible possibility that would accommodate the observed differences in shoulder abundance is that there is a slower fork progression through the mAdA-C region, which would allow for the mAdA-C and -I origins to have similar strengths in their peak regions, but different shoulder abundance, as observed here.

Analysis of protein–DNA origin interactions using NEs indicated an origin-specific association, as demonstrated by competitive EMSA (Fig. 2). This complex was competed by the anti-Cdc6 antibody (Fig. 3), thus authenticating these sequences as bona fide origins able to recruit Cdc6 in vitro. Furthermore, this interference was not observed when the origin-lacking mAdA-D DNA fragment was used, indicating sequence-specific interaction of Cdc6 with the origins. More interesting was the apparent complete absence of protein-origin DNA complex formation in EMSAs where the extracts were treated with anti-Ku80 antibody (Fig. 4A–C), suggesting a requirement of Ku for formation of the complex. The specificity of the antibody was confirmed using a blocking peptide and by the absence of detectable Ku80 in Ku80$^{-/-}$ extracts. These results are consistent with previous reports of Ku's role in stabilizing initiator proteins at origins, including the ORC complex in vitro in *S. cerevisae* [Feldmann et al., 1996] and in vivo in human HCT116 cancer cells [Sibani et al., 2005b]. To date, the region of Ku80 involved in origin binding and DNA replication has yet to be elucidated. Data provided by the C-terminus targeting anti-Ku80 antibody (Fig. 4) suggest that this function may reside within that domain. While the terminal 12 amino acids have been reported to interact with the DNA-binding protein kinase (DNA-PK), there is a complete lack of evidence for a DNA replication function for DNA-PK. In fact, unlike Ku80-deficient cells, DNA-PK deficient SCID cells grow faster than wildtype cell [Watanabe et al., 2003]. Consequently, it may be that the C-terminal domain of Ku80 has a role in DNA replication independent of DNA-PK, and further studies are required to uncover it.

In contrast to human cells, a deficiency of Ku80 in the murine embryonic fibroblasts (MEFs) used in this study did not decrease origin activity, either episomally (Fig. 6) or in situ (Fig. 7). Unlike the primary Ku80$^{-/-}$ MEFs which displayed a prolonged doubling time, no decrease in cell growth and proliferation has been observed for these MEFs (data not shown), which are transformed by the SV40 large T-antigen [Nussenzweig et al., 1996]. Such a discrepancy may either be attributed to a difference in species and the inadvertent targeting

of the human KARP-1, a second protein transcribed from the Ku80 locus [Myung et al., 1997], or the possible unpredicted side effects of using SV40 T-antigen transformed MEFs. $Ku80^{-/-}$ MEFs that were not transformed with T-antigen were very difficult to culture and grew at a prohibitively slow rate to permit proper experimentation (unpublished data).

Sequence analysis of both amplicons revealed the presence of homologs of the yeast *ARS* consensus sequence (ACS)(5′-$^{A}/_{T}$TTTA$^{T}/_{C}{}^{A}/_{G}$ TTT$^{A}/_{T}$-3′) (Fig. 8) [Deshpande and Newlon, 1992; Rivier and Rine, 1992; Huang and Kowalski, 1993]. Amplicon mAdA-1 contained a segment with 82% identity to ACS (9/11 homologous bases), while mAdA-C had a one-nucleotide deletion and one mismatch, also producing an 82% identity to ACS (9/11 homologous bases). Moreover, both amplicons contained potential DNA unwinding elements (DUE) as predicted by the WEB-THERMODYN program [Huang and Kowalski, 2003]. DUEs were previously shown to be important in initiation of DNA replication [Huang and Kowalski, 1993; Liu et al., 2003]. Furthermore, mAdA-1 contained binding sites to various transcription factors, including octamer 1 (Oct1) which has been shown to enhance DNA replication in vitro, possibly by mediating protein–protein interactions [Iguchi-Ariga et al., 1993; Matheos et al., 1998]. Another transcription factor binding site within the vicinity of the ACS is that of E2F1. Recent reports of a tight association between Ku80, E2F4, and CDK4 [Gullo et al., 2006] prompt the examination of any potential associations between Ku and E2F1, especially since episomal DNA replication assays in mammalian cells showed that E2F, Oct1, AP1, and HSE transcription factors stimulate DNA replication [Yokoyama et al., 1997]. Finally, two binding sites for the oncogene JUND are present in mAdA-1. JUND is the most broadly expressed member of the JUN family of transcription factors and in its absence primary murine fibroblasts undergo a p53-dependent cell cycle arrest and premature senescence. The exact mechanism by which these transcription factors enhance DNA replication remains unknown, but may include recruitment of the DNA replication machinery through protein–protein interactions, modulation of chromatin structure, mediation of nuclear matrix attachment of the origins, or by regulating transcription, thereby affecting

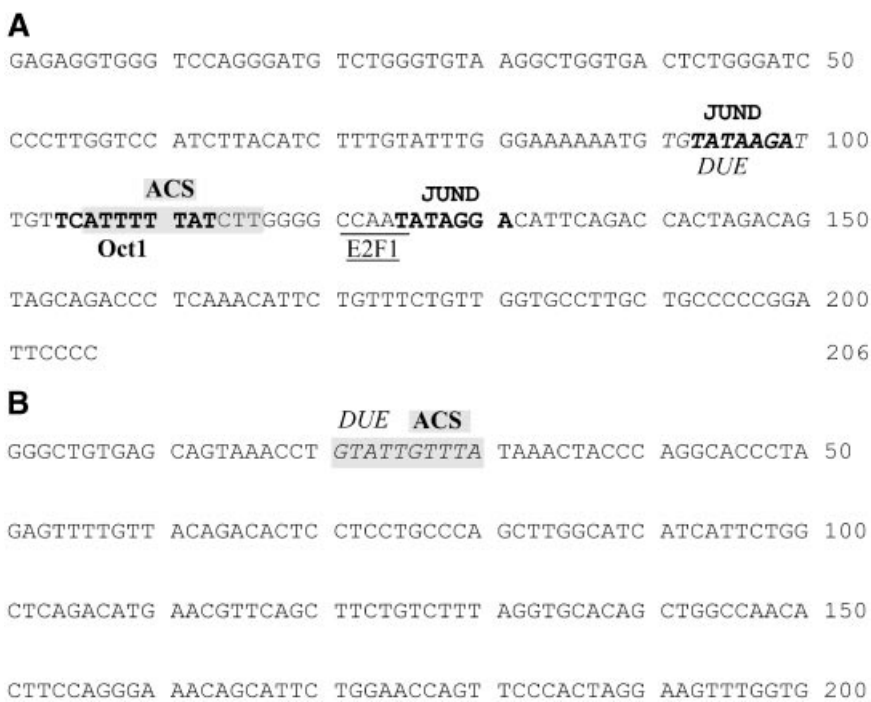

Fig. 8. Sequence characteristics of the two mAdA origins -1 (**A**) and -C (**B**). The location of transcription factor binding sites for JUND, Oct1, and E2F1 are indicated. Regions with 82% identity to the ARS consensus sequence (ACS) are highlighted in gray. The locations of putative DUE elements, as determined by the WEB-THERMODYN program, are indicated by the italicized sequence.

DNA replication (reviewed in [Murakami and Ito, 1999]).

In conclusion, this is the first study to fine-map the late-firing mAdA region in embryonic fibroblasts. The presence of two origins is apparent, but it is not known whether both fire within the same cell. The two origins identified were able to recruit the DNA replication initiator proteins Cdc6 and Ku80 in vitro and confer autonomous replication activity on their plasmids. Their activities, however, were not influenced by the absence of Ku80, either episomally or in situ. This fine mapping will enable an analysis of the specific sequences required for DNA replication and identification of their binding proteins.

ACKNOWLEDGMENTS

This research was supported by grants from the CIHR (M.Z-H.). S.S. was a recipient of a doctoral studentship from the CIHR/K.M. Hunter Charitable foundation and the FCAR/FRSQ, as well as the McGill Medicine studentship. E.R. was the recipient of a CIHR Cancer Consortium training award and the Max Stern Fellowship from McGill University. D.D.P. was a recipient of a CIHR Cancer Consortium training award.

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